UNIVERSITY OF TASMANIA

The genetic control of vegetative phase change in pea

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Jacqueline K. Vander Schoor

09 February 2018

Abstract

Plants exhibit numerous changes as they develop from a germinating seed through vegetative and reproductive stages to maturity. A seedling begins in a juvenile vegetative phase, and grows in size through the addition of new vegetative organs. As the plant acquires reproductive competence it enters an "adult" vegetative phase where flowering can occur in response to favourable environmental conditions. The initiation of reproductive structures signifies transition to the adult reproductive phase, in which gametes can eventually be produced and the formation of new seeds can occur, followed by senescence and death or a period of dormancy that may involve reversion to the adult vegetative phase. In order to maximise reproductive outputs, the life cycle of the plant and timing of development needs to be finely tuned and flexible enough to take advantage of favourable environmental conditions. The phases of plant development are thus tightly controlled genetically, but also have the ability to respond to the environment to some extent.

This study used the model legume pea (*Pisum sativum* L.) to investigate genes with a possible role in developmental timing, in order to improve our understanding of the genetic control of developmental timing in pea and temperate legumes generally. It will address the following questions: What might the vegetative phase transition look like in pea and how is it related to the timing of flowering in the adult reproductive phase? Can mutants affecting vegetative phase change be identified in pea and if so, how do these mutants interact and what is their molecular nature? Finally, what are the composition and phylogenetic structure in pea of gene families for key Arabidopsis phase change genes, and is there any evidence that any of these genes could participate in phase change in pea?

This research has made use of previously identified mutants, *aeromaculata 1* (*aero1*) and *aero2* and describes two new mutants, *accelerated phase change 1* (*apc1*) and *apc2*, which all exhibit pleiotropic defects in various aspects of plant development. However, their most interesting common feature is an acceleration in the normal progression of compound leaves from simpler to more complex structure, such that mutants display more complex leaf structure earlier in development than the isogenic wild-type plants. This may indicate a shift in the timing of vegetative phase change in these mutants. The largely additive, but in some cases synergistic effects of these mutants in combination suggest that these genes have overlapping roles in

control of several processes in plant development, including not only timing of vegetative development/compound leaf morphology, but also flower development and fertility, pod development, and phyllotaxy.

Efforts were also made to improve or establish map positions for three of the four loci; *AERO1*, *AERO2* and *APC1*. The *AERO1* locus mapped close to the pea ortholog of *CURLY LEAF* (*CLF*), a gene with known roles in flowering time control in Arabidopsis. Sequencing revealed mutations in the *CLF* coding sequence in two independent *aero1* mutant alleles, indicating *CLF* and *AERO1* are likely to be the same gene. The known role of Arabidopsis CLF in epigenetic regulation of gene expression and the pleiotropic effects of *AERO1* are consistent with the idea that *AERO1/CLF* may operate as a master regulator of all aspects of plant developmental timing and might do this by modifying the activity of specific genes involved in phase change, since *aero1* showed an acceleration of the timing of all phases of development, including vegetative development and flowering. For *apc1*, fine mapping and an RNA-sequencing approach were used to identify a potentially causal mutation in the ortholog of Arabidopsis FTSH11. This gene is not previously known to play a role in developmental timing in other species, so may prove to have a novel role in legume development.

Genetic control of phase change in plants is achieved through the highly conserved miRNA156-SPL module. The final part of this study isolated, annotated and characterised the miR156 and SPL gene families in pea using newly-available genomic resources. This resulted in the identification of most but not all of the family members predicted from comparative analyses with *Medicago truncatula*. Of the pea miR156 precursor sequences isolated, none appeared to be obviously involved in vegetative phase change based on their expression patterns. However, investigation of expression patterns for *SPL* genes revealed several that are developmentally regulated in wild-type pea in a manner consistent with a possible role in regulation of phase change in pea.

Overall, these findings represent an important contribution to the knowledge of developmental timing in pea and related legumes, the relationship between compound leaf development and vegetative phase change, and the genes that may be crucial for the control of these processes.

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Table of Contents

Chapter 1

Introduction & Literature Review

1.1 The Phenomenology of Phase Change

1.1.1 The history of the idea of phase change

Plants pass through phases of development from embryo through to maturity. The sequence of these events is called ontogeny and the precise timing of these events is crucial for a successful life cycle. Observing the ontogenetic features of plants has been of interest for centuries, with the first insights of plant growth phases documented in the eighteenth century (Goethe, 1790; Knight, 1795). A century later, Goebel (1889) coined the phrase "heteroblastic" to describe the dramatic variation observed between juvenile and adult plants of the same species, as exemplified by ivy (Rogler & Hackett, 1975), Acacia (Kaplan, 1980) and certain species of Eucalyptus (Jordan *et al.*, 1999; Wiltshire *et al.*, 1991). Since Goebel, many of the morphological, anatomical and physiological aspects of ontogeny have been documented and reviewed (Troll, W., 1939; Ashby, 1948; Allsop, 1967; Gatsuk *et al.*, 1980; Hackett, 1985).

1.1.2 Defining and identifying the phases of development

Modern descriptions of plant post-embryonic development routinely distinguish three discrete and temporal phases of growth – the juvenile vegetative phase, adult vegetative phase and the adult reproductive phase (Poethig 1990). The ***juvenile vegetative phase*** begins as a germinating seedling initiates a stem, leaves and axillary buds, and at this stage the plant is unable to flower. As the plant shifts to the ***adult vegetative phase***, morphological changes occur as the plant increases in stature and adds further vegetative nodes and becomes competent to respond to appropriate inductive conditions by initiating reproductive development. Finally, the plant finishes its developmental cycle in the ***adult reproductive phase***, in which flowers develop and gametes are produced, before eventual senescence and death. The transition between these phases is referred to as phase change and the modern use of the term heteroblasty has now been broadened to refer to any morphological variation in the vegetative shoot that occurs during ontogeny.

In higher plants, the transition to the adult reproductive phase is obviously marked by the production of specialised floral organs. In contrast, the transition from the juvenile vegetative to the adult vegetative phase is less clear. Apart from reproductive competency, vegetative phase change can be variously manifested in a range of other traits in different species including stem elongation, phyllotaxy, growth habit, lateral branching, photosynthetic capacity, rooting ability and disease and insect resistance (Schaffalitzky de Muckadell, 1954; Doorenbos, J., 1965; Allsop, 1967). However, one organ type whose morphology changes through development to some extent in all vascular plants is leaves. These vital plant structures exhibit developmental plasticity as they adapt to both external and endogenous cues, but also exhibit developmental changes associated with the transition from juvenile to the adult form in a coordinated, predictable and timely fashion prior to flowering. The pattern of ontogenetic change in leaf anatomy and physiology appears to be inherent and genetically determined, although the transition can be slowed or reversed by environmental conditions such a poor nutrition, defoliation, water stress, low light and low temperatures (Allsop, 1967).

The vast diversity across plant species has meant that the visible nature of phase change may be quite distinct in different species. In Arabidopsis, vegetative phase change is manifested in relatively subtle and gradual change in the shape of the leaf and the appearance of trichomes on the abaxial leaf surface (Telfer & Poethig, 1994; Chien & Sussex, 1996). In maize, the juvenile and adult vegetative phases are distinguished by leaf epicuticular wax type, epidermal hairs and epidermal cell shape, among other features (Moose & Sisco, 1994). In contrast, the woody species English ivy exhibits a sudden and dramatic change in leaf shape from multi-lobed to heart shaped (Robbins, 1957; Rogler & Hackett, 1975) and many Eucalyptus species switch from sessile, obtuse and glaucous leaves in the juvenile plant to petiolate, lanceolate and shiny green in the adult (James & Bell, 2001). In general, juvenile leaves tend to be smaller and simpler, with adult leaves being larger and more complex. Even in plants that always produce simple leaves, such as Arabidopsis, subtle changes can be seen in traits like serrations, petiole length or length to width ratio (Telfer & Poethig, 1994). Some of the most striking examples are those species where the adult's leaves undergo modification to form spines (*Ulex europaeus*) or phyllodes (Acacia) (Bieniek & Millington, 1967; Kaplan, 1980). This diversity exhibited across the plant kingdom leads to complexities in identifying and defining vegetative phase change.

1.1.3 The complexities of vegetative phase change

In an analysis of more than one hundred plant species, Gatsuk (Gatsuk *et al.*, 1980) arrived at the conclusion that variation was more complex than a standard three-phase categorization, and proposed the existence of nine phases of growth, including several intermediary vegetative forms, that could be used to classify most plant species. The first few leaves formed after germination in the early juvenile phase, provide an illustration of this. These leaves are often distinct from any other leaves on the plant. For example, in maize the first leaf is small and elliptical with a blunt end, but the subsequent juvenile leaves are lanceolate and pointed. (Bongard-Pierce *et al.,* 1996). In soybean, the first two leaves produced after the cotyledons are simple and opposite, whereas the third and fourth juvenile leaves are trifoliate and frequently distichous (Yoshikawa *et al.*, 2013). Some plants also exhibit intermediate forms between the juvenile and adult leaf. In the strongly heteroblastic tree from New Zealand, *Pseudopanax crassifolius*, eight different types of leaves are produced in the seedling, juvenile, transitional and adult phases of growth (Gould, K.S., 1993).

A significant amount of research has been undertaken in different species to characterize the external morphological features that may be clear indicators of phase change. Dramatically heteroblastic species can create the misleading impression that phase change is discrete and simple. Although early research focused on particular species in which this was true, subsequent research including in many model species such as Arabidopsis, the changes have been found to be more subtle and gradual. The reality seems to be that variation in vegetative organs during shoot development is far more likely to be continuous and gradual, with a trajectory of gradual change in vegetative traits that starts in the juvenile phase and continues through until senescence. It follows that the transition from the juvenile to the adult vegetative phase occurs somewhere along that continuum before reproduction and that the nature and duration this period of transition may be different in different plant species. This makes identification of common universal features and comparisons difficult, and has also raised the question of whether there are universal genetic and molecular mechanisms that might underlie and explain the diverse morphological and physiological manifestations of vegetative phase change.

1.2 The Genetic Control of Phase Change

1.2.1 Early genetic research

Early discoveries of the mechanisms that controlled phase change came through the isolation of mutants that modified developmental patterns. Maize semi-dominant mutants, *Corngrass* (*Cg*), *Teopod 1* (*Tp1*), *Teopod* 2 (*Tp2*) and *Teopod 3* (*Tp3*) were some of the earliest identified to have profound effects on vegetative morphology, including reversion from the adult to the juvenile vegetative phase and the transformation of reproductive structures into leaves. These mutants suggested that the traits expressed during the juvenile phase may be part of a genetically regulated developmental program (Galinat, 1966; Poethig, 1988). Phase change in Arabidopsis was also first characterized at around the same time, and its genetic control initially explored through mutants that affected the timing of changes in leaf trichome distribution that occur during development (Telfer *et al.*, 1997). Study of various flowering time mutants revealed an uncoupling of these changes from the flowering transition and led to the conclusion that additional components unique to the regulation of each of the different phases must exist (Telfer *et al.*, 1997).

1.2.2 The miRNA156-SPL module regulates vegetative phase change

With the advent of molecular techniques and genomic resources for many plant species, progress in understanding the underlying genetic control of phase change has developed rapidly. In particular, two microRNAs (miRNAs), miR156 and miR172, are now considered to be central components in a conserved pathway for regulation of phase change. These were first identified in *Arabidopsis* and maize (Chuck et al., 2007a; Wu and Poethig, 2006), and have subsequently been shown to play similar roles across a wide range of herbaceous and woody plants (Xie, 2006; Wang *et al.*, 2011; Zhang *et al.*, 2011; Shikata *et al.*, 2012; Bergonzi *et al.*, 2013; Zhou, *et al.*, 2013). This points to the existence of a common mechanism that determines the timing of vegetative phase change and underlies its diverse morphological manifestations. In juvenile plants, expression of miR156 is high and subsequently declines during vegetative phase change (Wu & Poethig, 2006; Wang, Czech, *et al.*, 2009). Overexpression of miR156 can prolong the juvenile phase and delay flowering in both monocots and dicots (Schwab et al., 2005; Wu and Poethig, 2006; Xie, 2006; Chuck et al., 2007a; Zhang et al., 2011; Aung et al., 2014; Wang et al., 2015; Chen et al., 2015; Cao et al., 2015). Conversely, blocking miR156 action in Arabidopsis promotes vegetative phase change and results in plants with a precocious adult phenotype (Franco-Zorrilla *et al.*, 2007; Wang, Czech, *et al.*, 2009; Wu *et al.*, 2009).

MiR156 targets a subset of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPB/SPL*) transcription factors that possess distinct functions necessary for vegetative phase change (Cardon *et al.*, 1999; Schwab *et al.*, 2005), and suppresses *SPL* expression via transcriptional cleavage, so as miR156 declines with age, *SPL* expression increases. SPL proteins were first discovered in *Antirrhinum majus* as transcription factors that interacted with the promoter of the floral meristem identity gene *SQUAMOSA*, a MADS domain transcription factor orthologous to Arabidopsis *AP1* (Huijser *et al.*, 1992; Klein *et al.*, 1996). In Arabidopsis, there are 17 *SPLs*, of which 11 are regulated by miR156. Independent expansion of the *SPL* gene family in most species studied has presented challenges for functional analysis. The targeting of specific SPLs by miR156 remains conserved, but the roles appear to have specialised and redundancy is prevalent (Guo *et al.*, 2008; Yang *et al.*, 2008; Preston & Hileman, 2013).

In Arabidopsis, the functional significance of the miR156-SPL interaction has been well documented. Overexpression mutants of miR156-resistant SPLs result in accelerated vegetative phase change and premature flowering, similar to mutants in which miR156 action is blocked through target mimicry, where a non-cleavable RNA forms a non-productive interaction with the complementary miRNA156 target sites in SPLs (Franco-Zorrilla *et al.*, 2007). Conversely, when loss-of-function mutations in SPLs were generated, this delayed vegetative phase change (Cardon *et al.*, 1997; Wu & Poethig, 2006; Gandikota *et al.*, 2007; Schwarz *et al.*, 2008; Wang, Czech, *et al.*, 2009; Xu, Hu, *et al.*, 2016). Further, the miR156-SPL pathway appears to be a conserved master switch in heteroblasty, even with the striking morphological diversity observed among species. This is typified by the temporal control of trichome distribution in Arabidopsis (Yu *et al.*, 2010; Xue *et al.*, 2014), the age-dependent regulation of leaf complexity in *Cardamine hirsuta* (Rubio-Somoza *et al.*, 2014) and control of juvenile to adult leaf cell morphology in maize (Chuck et al., 2007).

1.2.3 The SPL-miR172 module regulates reproductive phase change

Specific *SPL* genes in turn promote the transcription of miRNA172, which also increases with age and thus has an opposing expression pattern to miR156 (Wu *et al.*, 2009). MiR172 then targets a number of AP2-like transcription factors that act as repressors of the flowering transition through inhibition of FT (Cardon *et al.*, 1997; Aukerman, 2003; Mathieu *et al.*, 2009; Jung *et al.*, 2014). MiR172 acts through both modes of mRNA cleavage and translational repression, but the latter is more common (Aukerman & Sakai, 2003; Chen, X., 2004; Schwab

et al., 2005; Zhu & Helliwell, 2010). The interaction between miR172 family and its AP2 or AP2-like targets is deeply conserved in flowering plants and has been functionally tested in a number of species including rice, maize, barley and potato (Aukerman and Sakai, 2003; Lauter et al., 2005; Chuck et al., 2007b; Nair et al., 2010; Lee et al., 2014). When miR172 is overexpressed in Arabidopsis (35S::miR172), plants undergo precocious vegetative phase change and early flowering. Similarly, plants in which the miR172 targets *TOE1* and *TOE2* have been mutated show earlier expression of adult traits. By contrast, both the miR172 knockout mutant and the *35S::TOE1* plants showed a delay in all phases of plant development (Aukerman & Sakai, 2003; Jung *et al.*, 2007; Wu *et al.*, 2009).

Under normal circumstances, the transition from juvenile to the adult vegetative phase must occur before a plant can become reproductively competent. The miR156-SPL-miR172 pathway establishes that the plant is now mature enough to reproduce (Figure 1). There are, however, numerous other floral induction pathways that work in parallel with each other that feed into the same integrator genes. These include both endogenous pathways (hormone signalling and carbohydrate assimilation) & environmental pathways (temperature, photoperiod, light quality & stress). In some instances, the genes that regulate flowering through these other pathways have no effect on vegetative phase change suggesting that these transitions can be uncoupled (Wiltshire *et al.*, 1994; Telfer *et al.*, 1997; Wiltshire *et al.*, 1998), but recent research is uncovering new connections between the pathways that increase the complexity of phase transitions, their genetic control & interactions (Zhu & Helliwell, 2010; Wang & Wang, 2015).

1.2.4 Additional genetic factors controlling phase change

In the last two decades, research has identified a number of other genetic factors controlling the miR156-SPL-miR172 module, mostly through the analysis of Arabidopsis mutants showing either a precocious or delayed onset of adult traits. CENTER CITY and GRAND CENTRAL are core components of the Mediator complex that influences transcription of mRNAs and miRNAs at all developmental stages and were found to influence vegetative phase change through repression of miR156 (Kim *et al.*, 2011; Gillmor *et al.*, 2014; Yin & Wang, 2014). Several genes acting in posttranscriptional silencing pathways have also been implicated in vegetative phase change; including Dicer-like genes (Gasciolli *et al.*, 2005; Xie *et al.*, 2005; Yoshikawa, 2005), the exportin 5 ortholog, HASTY (Telfer & Poethig, 1998; Bollman, 2003),

the zinc-finger-domain protein SERRATE (Clarke *et al.*, 1999); an AGO-family member, ZIPPY (Hunter *et al.*, 2003), SUPRESSOR OF GENE SILENCING3 and RNA-DEPENDENT POLYMERASE6 (Peragine, 2004). Further, research into genes that modify gene expression through epigenetic regulation has also revealed factors that specifically control the expression of miR156. BRAHMA, a chromatin remodelling ATPase, and SWINGER, a component of the Polycomb Repressive Complex 2, act antagonistically on miR156 to regulate vegetative phase change (Xu, Guo, *et al.*, 2016). Further, SWINGER may work synergistically with a second chromatin remodeler, PICKEL, to repress miR156 (Xu *et al.*, 2015). Plants with mutations in the cytosine DNA methyl-transferase gene, MET1 also exhibit a heterochronic delay in the juvenile to adult leaf transition (Kankel *et al.*, 2003). So far, these genes controlling the phase change pathway have been identified as part of major gene regulatory, processing or biogenesis pathways and, not surprisingly, exhibit pleiotropic defects as they are involved in many different processes. These reveal a complex and multi-level control over vegetative phase change. Whether they are conserved across the plant kingdom, and how they might specifically operate on the miRNA156-SPL-miR172 pathway is yet to be determined.

1.3 Environmental & Endogenous Control of Phase Change

1.3.1 Early studies

Initial studies attempting to determine which part(s) of the plant are responsible for the control of phase change demonstrated that defoliation or severe pruning prolonged the juvenile phase in both woody and herbaceous plants, suggesting the action of a shoot-derived factor (Schaffalitzky de Muckadell, 1954; Njoku, 1956; Libby & Hood, 1976). Further support for this conclusion was provided by various grafting studies performed on Eucalyptus, conifers and fruit trees (Schaffalitzky de Muckadell, 1954; Wareing, 1959; Wiltshire & Reid, 1992). In particular, the reciprocal grafting of *Eucalyptus tenuiramis*, which undergoes vegetative phase change around node 36 and its sister species *Eucalyptus risdonii*, which remains permanently juvenile, demonstrated the that the timing of the vegetative phase change mechanism is indeed localised to the shoot (Wiltshire & Reid, 1992). Grafting adult shoots from *E. tenuirami*s onto *E. risdonii* root stock or juvenile shoots of *E. risdoni*i onto *E. tenuiramis* root stock had no effect on the timing of vegetative phase change in the scions. Further studies in maize, using cultured shoot apices also showed that phase specific epidermal traits in leaves are determined after leaf initiation, acting directly on leaf primordia, rather than the apical meristem (Orkwiszewski & Poethig, 2000). More recently, Yang et. al. (Yang *et al.*, 2011) removed either leaf primordia, cotyledons or the root system from young Arabidopsis seedlings and found that only the removal of leaf primordia delayed phase change and this was associated with an increase in miR156 expression.

Another interesting discovery made by Wiltshire & Reid (1992) in their study on Eucalyptus, was that vegetative phase change correlated with the amount of light that the shoot received, rather than day length or temperature. A reduction in photosynthetic rate is also known to prolong the juvenile phase in tobacco plants (Tsai *et al.*, 1997; James & Bell, 2000) and the same is observed in Arabidopsis photosynthetic mutants (Yu *et al.*, 2013). In concert, these early studies suggested that the timing of vegetative phase change was controlled by factors in the leaves that were light dependent.

1.3.2 Regulation of vegetative phase change by sugar

According to Goebel's original hypothesis (1908), leaves produced early in shoot development are typically small and morphologically simple because leaf development is arrested by low endogenous nutrient levels. Leaves then become more complex and larger as the metabolic

capacity of the plant increases. This hypothesis was confirmed by some of the earliest experiments in juvenility showing that the nutritional status of the plant did impact the duration of the juvenile phase and heteroblastic features (Goebel, 1908; Ashby, 1948; Allsop, 1954; Sussex & Clutter, 1960; Feldman & Cutter, 1970). This led to the idea that the endogenous signal controlling vegetative phase change may be sugars, a key product of photosynthesis. A number of sugars have since been shown to facilitate the juvenile to adult phase change by repressing miR156 expression, including glucose, sucrose, fructose and maltose (Wahl *et al.*, 2013; Yang *et al.*, 2013; Yu *et al.*, 2013). Further, when the enzymes involved in starch metabolism, sugar signalling or synthesis pathways such as HEXOKINASE1 (HXK1) or TREHALOSE PHOSPHATE SYNTHETASE 1 (TPS1) are knocked out, plants exhibit elevated levels of miR156, reduced expression of miRNA156 regulated *SPLs*, and delayed phase change (Chen *et al.*, 2004; Matsoukas *et al.*, 2013; Yang *et al.*, 2013; Yu *et al.*, 2013). Therefore, as the juvenile plant ages, sugars produced by photosynthesis accumulates, repressing miR156. This results in an increase in *SPL* expression that then promotes the shift to the adult phase (Proveniers, 2013; Yang *et al.*, 2013; Yu *et al.*, 2013).

1.3.3 Hormonal regulation of phase change

Another endogenous signal that has garnered interest over the years is the phytohormone gibberellin (GA), which plays diverse roles in plant growth and development, including seed germination, stem and leaf morphology and flowering time. The role of GAs has also been observed in the regulation of both phase change and phase specific traits. In different species, GA can induce adult shoots to revert to the juvenile phase (Rogler & Hackett, 1975; Zimmerman *et al.*, 1985) and has shown to accelerate vegetative phase change and flowering in other species (Evans & Poethig, 1995; Mimura *et al.*, 2012; Telfer *et al.*, 1997). GA levels do not rise in the shoot in early seedling development and have no effect on the expression of miR156 in Arabidopsis, but do promote the expression of specific *SPLs* associated with phase change (Eriksson, 2006; Jung *et al.*, 2012).

A mechanism for this action was recently demonstrated, in which GA acts through DELLA proteins that function to suppress *SPLs*, but GA-mediated degradation of DELLA allows SPLs to be upregulated and activate the floral transition through miR172 (Galvao *et al.*, 2012; Yu *et al.*, 2012; Hyun *et al.*, 2016). Considering this lack of direct control, it is likely that GAs do not play a central role in vegetative phase change, but may be important for

integrating other signals. Other research suggests that phase change could also be regulated by other hormones that may work in conjunction with GA, or interact with DELLA proteins also, such as abscisic acid (ABA), auxin (AUX), cytokinins (CK) and jasmonic acid (JA) (Chaudhury *et al.*, 1993; Gazzarrini *et al.*, 2004; Fahlgren *et al.*, 2006; Hibara *et al.*, 2016), Due to the nature of hormones, their involvement in many different processes and their high degree of cross-talk, this area of research is still expanding and many questions still need answering.

1.3.4 Environmental regulation of phase change

So far, understanding of environmental inputs that regulate vegetative phase change has been minimal, with only a few isolated and unrelated studies addressing this question. A comprehensive initial study by Telfer et. al. (Telfer *et al.*, 1997), showed that Arabidopsis plants grown in SD exhibit a delay in vegetative phase change, indicating a role for photoperiod in this process. Further, some Arabidopsis flowering time mutants also showed vegetative phase change defects such as *constans* (*co*; photoperiod pathway), *gigantea* (*gi*; circadian clock) and *fd* (vernalisation pathway). Interestingly, not all late flowering mutants showed a previous delay in the juvenile phase, indicating that vegetative and reproductive timing have some regulatory factors in common, but that these phases in plant development can be separately controlled (Telfer *et al.*, 1997). Interestingly, GI has also been shown to directly regulate miR172 levels in Arabidopsis to promote flowering in a CO-independent genetic pathway (Jung *et al.*, 2007). Other, unrelated studies on the particular environmental factors that control phase change have shown that elevated CO_2 levels accelerate flowering in Arabidopsis, in part due to a decrease in miR156 and increased levels of *SPLs* (May *et al.*, 2013). Conversely, elevated temperatures cause increased miR156 expression and suppression of *SPL*s, indicating that ambient temperature may also be an input signal (Lee *et al.*, 2010; Kim *et al.*, 2012).

Overall, these studies lead to the conclusion that a range of environmental and endogenous factors may influence the conserved miR156-SPL-miR172 module and therefore its regulation phase change (Figure 1). However, there are clearly many questions that still need answering, particularly in view of the fragmented nature of the work on this topic so far. This makes it both challenging for research, to understand how it is regulated, but also provides potential possibilities for future research. In particular, manipulating the miR156-SPL-miR172 pathway

in plants will provide novel ways to regulate the timing of development and phase specific traits potentially useful for agronomic production (Wang & Wang, 2015).

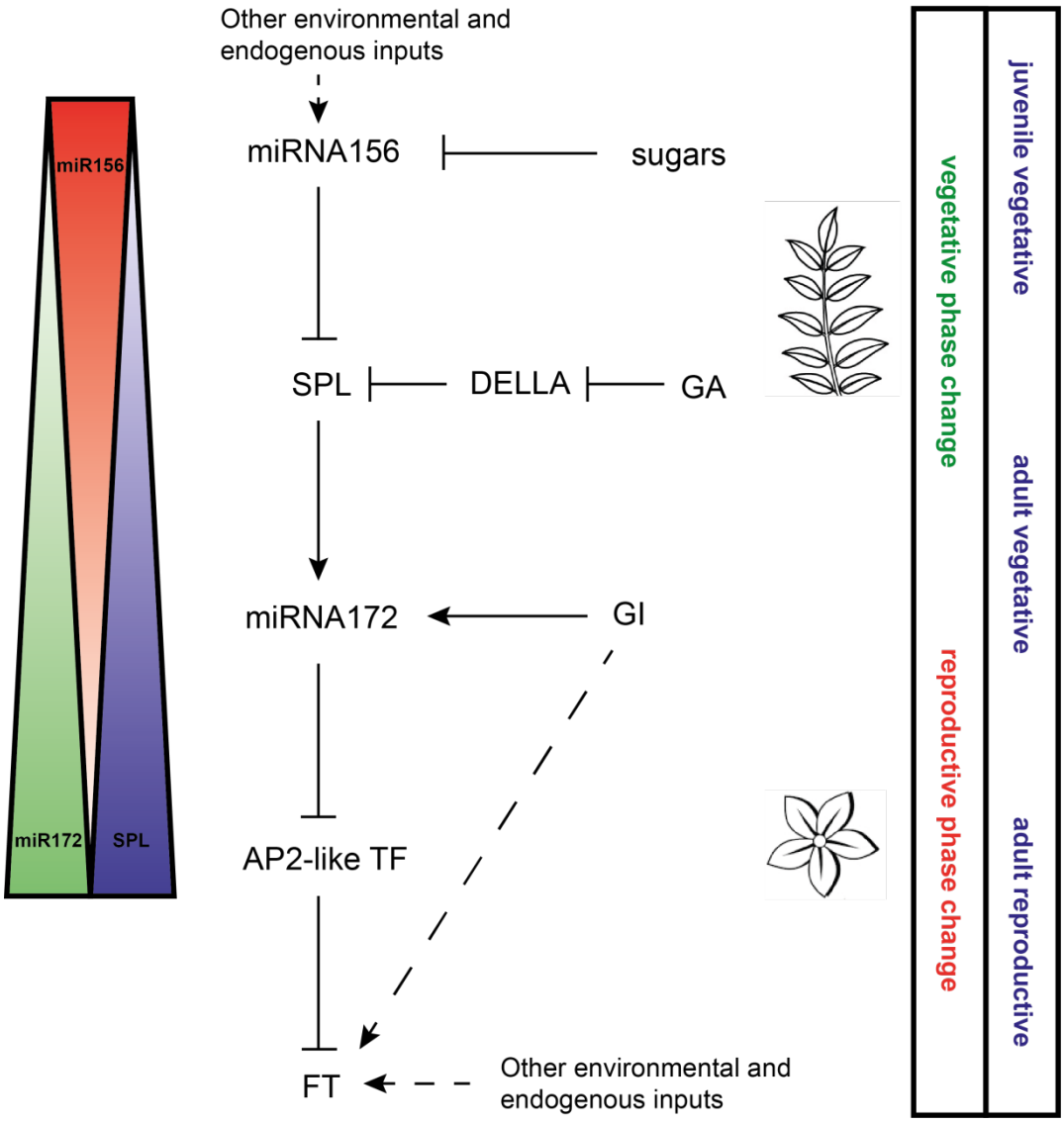


Figure 1. Overview of phase change in plants

There are two major phase changes in plant development after germination. The vegetative phase change (juvenile vegetative to adult vegetative) and the reproductive phase change (adult vegetative to adult reproductive). The major known inputs into this pathway have been indicated. The vertical gradual shadings for miR156, miR172 and SPL indicate expression level changes during the developmental process. A line with an arrow head indicates positive regulation, whereas a line with a bar at the end indicates suppression. A solid line means direct effect, dashed line means unknown/indirect effect.

1.4 Phase Change in Legumes

Legumes (Fabaceae) are the third largest family of higher plants with close to 20,000 species, and are second only to grasses in agricultural importance (Doyle, 2001; Graham, 2003). Some of the major legumes have also been used for detailed genetic analysis and functional studies, including soybean (*Glycine max*), medicago and pea. Although much work has been done on the transition to flowering in these model legumes, very little effort has been devoted to understanding vegetative phase change. Many legume species undergo distinct vegetative changes as they mature, and among these, compound leaf structure is one trait of particular note, since it changes through the juvenile and adult phases. Leaves in the Fabaceae family are often pinnately compound (although occasionally trifoliate or palmate), and increases in size or changes to leaflet patterning or positioning occurs as the plant matures. Along with readily available mutants and recent improvements in genomic resources, this makes them an ideal plant group to study the phenomenon of vegetative phase change.

Initial reverse genetics studies in legumes have confirmed the presence and function of the miR156/SPL/miR172 pathway. In soybean, the complementary expression patterns of miR156 and miR172 are conserved (Yoshikawa et al. 2013). Yamashino et al. (2013) found that overexpressing *Lotus japonicus* miR172 in Arabidopsis resulted in accelerated flowering through increased expression of FT. More recently, research was done using overexpressed miR156 mutants in *L. japonicus* (Wang *et al.*, 2015) and *Medicago sativa* (Aung *et al.*, 2014). They both showed multiple defects including a delay in vegetative development and flowering time. This confirms that there is conservation of the key components involved in phase change in legumes, but whether this pathway is connected to juvenile to adult vegetative development in these species, and how it is regulated remain to be uncovered.

Among legumes, pea is a well-known model for genetic analysis of developmental processes. These include hormone function, flower initiation and development, leaf development, seed development and root symbioses, and many genes controlling these processes have been cloned through the isolation and characterization of relevant mutants. The control of the transition from vegetative to the reproductive phase has been well studied, and a number of different homologues of Arabidopsis floral genes have been discovered (Hecht *et al.*, 2005). However, little research has been undertaken to determine the exact morphological changes that occur in

the juvenile to adult phases of pea vegetative development and how they are regulated genetically.

Pea plants produce compound odd-pinnate leaves that display changes in morphology during ontogeny (Allsop, 1967). In most genetic backgrounds, the first two leaves are reduced “scale” leaves and the first true leaf (leaf 3) consists of a pair of large, foliaceous stipules, and a rachis bearing one pair of leaflets and a terminal tendril. The number of lateral organ pairs increases with age, usually reaching a maximum of three leaflet pairs and three lateral tendril pairs at around the node of first flower (Barber, 1959; Gould *et al.*, 1992). The timing of compound leaf development during maturation in pea is the most distinct vegetative feature of the plant and may be a heteroblastic indicator of vegetative phase change. This was first noted by Barber (Barber, 1959) when he discovered that the node at which the number of leaflets changed from two to four varied among different pea varieties, and that some late flowering varieties also had an associated delay in the change to four, and subsequently, six leaflets.

The first insights into the genetic control of vegetative phase change in pea were provided by (Wiltshire *et al.*, 1994), who examined the relationship between leaflet number and flowering time in a number of previously characterised flowering mutants (Murfet, 1978, , 1990; Murfet & Reid, 1993). This work showed that the nature of phase change was likely to be complex, with some genes controlling both leaf development and flowering time in parallel, and others only affecting flowering time, as confirmed later by the Arabidopsis research discussed above (Telfer *et al.*, 1997). Further work by (Taylor & Murfet, 2003; Murfet & Taylor, 2004) described two pea mutants, *aero1* and *aero2*, with pleiotropic phenotypes that included an acceleration in the timing of development as observed by an acceleration of compound leaf growth. The *aero1* mutant was also reported to have a small effect on flowering time, whereas *aero2* did not, providing an additional indication that this vegetative change can be uncoupled from flowering.

1.5 Research Objectives

Accumulating evidence suggests that understanding the pathways and genes that control vegetative phase change will provide important tools to optimise the timing of growth and development, particularly in important crop varieties such as legumes. Even though progress in understanding reproductive phase change in legumes has been substantial, understanding of both the molecular and physiological nature of vegetative phase change lag significantly behind current research in other species. This has, in part, been due to limited genomic resources, but recently this has shifted with the advent of sequencing projects in most of the major legume species (Gupta *et al.*, 2014; O'Rourke *et al.*, 2014).

This project will attempt to address this issue by investigating the genetic control of vegetative phase change in pea using new and previously described mutants. Further, using current genetic research in other model species such as Arabidopsis and maize as a guide, key gene families potentially involved in phase change in pea will be characterized and their regulation examined. The aim of this project will be to integrate both physiological and molecular data to improve our understanding of this crucial mechanism, not only in pea but applicable to the wider family of legumes. To achieve these aims, the following specific research activities have been undertaken:

1. Genetic and morphological characterisation of two new mutants with altered timing of changes in compound leaf complexity. Comparison with known mutants, *aero1* and *aero2*, and preliminary analysis of genetic interaction among these loci.
2. Investigation of the map positions of all four loci, and the identification and evaluation of candidate genes.
3. Identification and phylogenetic analysis of miR156 and *SPL* gene families in pea and preliminary analysis of their expression patterns.

Chapter 2

Materials and Methods

This chapter describes the general materials and methods used for all research presented in this thesis. For specific modifications or methodology associated with individual chapters there are materials and methods sections in those relevant chapters.

2.1 Plant materials

Details of the pea lines and mutants used for this research are outlined in the tables below (Table 2.1 and 2.2). Apart from *aero1-10*, each of the single mutants were introgressed into NGB5839 background through at least 3 backcrosses, and populations segregating single, double and triple mutants were created by crossing and growing F2 and F3 populations.

Table 2.1 Details of progenitor lines used in this study as wild-type lines and/or parental lines for mapping crosses.

Line	Description	References
NGB5839	Wild-type line originally used in mutagenesis programmes. Gibberellin deficient (*le-3*) dwarf of cv. Torsdag	(Lester *et al.*, 1997; Weller *et al.*, 2003)
cv. Torsdag (TOR)	Wild-type line originally used in mutagenesis programmes. Tall line from which NGB5839 is derived.	Hobart Line 107
cv. Térèse (TER)	Parental line for narrow mapping crosses with mutants in NGB5839 background	(Laucou *et al.*, 1998)

Table 2.2 Details of pea vegetative development mutants grown in this study.

Line	Details	References
aero1-1 (WL-5837; JI2767)	Created by EMS mutagenesis of cv. Torsdag and crossed into NGB5839	(Marx, 1986; Sidorova & Uzhintseva, 1995; Taylor & Murfet, 2003)
aero1-10 (MIII/12; WL-5880)	Created with gamma radiation in combination with 0.2% DS of cv. Virtus	(Blixt, 1962; Marx, 1986; Taylor & Murfet, 2003)
aero2	Created by EMS mutagenesis of NGB5839	(Weller, 1997; Murfet & Taylor, 2004)
apc1	Created by EMS mutagenesis of cv. Torsdag and crossed into NGB5839	Wiltshire, R. and Refli (unpublished)
apc2	Created by EMS mutagenesis of NGB5839	(Weller, 1997)

2.2 Plant growth conditions

For sowing, all seeds were coated in thiram (fungicidal powder) and sown in 14cm slim-line pots containing a 1:1 mixture of dolerite chips and vermiculite, topped with 3cm of native nursery grade potting mix with controlled release fertilizer (CRF) added (Horticultural and Landscape Supplies, Brighton, TAS, Australia). Plants were watered regularly and supplied with nutrient solution on a weekly basis. All plants described in this thesis were grown in controlled-environment growth cabinets or phytotrons at the University of Tasmania. Growth cabinets were used for all experiments in which highly accurate temperature or photoperiod controls were required. Growth cabinets were maintained at a temperature of 20°C and white light provided by cool-white fluorescent tubes (L40 W/20S cool white; Osram Germany) at an irradiance of 120-140 µmol m^{-2} s^{-1} unless otherwise specified. Temperature in the phytotron was maintained at approximately 24°C during the day and 16°C at night and plants were exposed to a base photoperiod of 8 hours of natural light in conjunction with darkness or extended lighting to create different photoperiod conditions.

2.3 Online sequence resources

Online sequence resources outlined in the individual chapters were used for identification, analysis of gene homologs and some primer design (See Table 2.3). Where genes were not annotated, or found to be annotated incorrectly based on expressed sequences or alignments between species, coding and protein sequences were corrected accordingly for use in phylogenetic analyses. Sequences for pea were either retrieved from the Transcriptome Shotgun Asssembly (TSA) available from GenBank (ncbi.nlm.nih.gov) (Franssen *et al.*, 2011; Kaur *et al.*, 2012), or more recently from the Pea RNA-Seq Gene Atlas (bios.dijon.inra.fr/FATAL/cgi.pscam.cgi) (Alves-Carvalho *et al.*, 2015).

Table 2.3 Online resources used for sequence information.

Species	Resource Site	Website	Reference
Arabidopsis thaliana	The Arabidopsis Information Resource (TAIR)	http://www.arabidopsis.org	(Lamesch *et al.*, 2012)
Brachypodium distachyon	Phytozome	https://phytozome.jgi.doe.gov/pz/portal.html	(Goodstein *et al.*, 2012)
Chickpea (*Cicer arietinum*)	The Chickpea Portal	http://www.cicer.info/databases.php	Prof. David Edwards (2016): Improved kabuli reference genome. CyVerse Data Commons. Dataset. http://doi.org/10.7946/P2G596
Common bean (*Phaseolus vulgaris*)	Phytozome	https://phytozome.jgi.doe.gov/pz/portal.html	(Goodstein *et al.*, 2012)
Maize (*Zea mays*)			
Medicago truncatula			
Rice (*Oryza sativa*)			
Soybean (*Glycine max*)			
Tomato (*Solanum lycopersicum*)			
Pea (*Pisum sativum*)	GenBank TSA Sequence Database PsCam Transcriptome Database	http://www.ncbi.nlm.nih.gov http://bios.dijon.inra.fr/FATAL/cgi/PsUniLowCopy.cgi	(Franssen *et al.*, 2011; Kaur *et al.*, 2012; Alves-Carvalho *et al.*, 2015)

2.4 Primer design

Primers were designed from pea sequence using the web based software Primer3 (primer3.wi.mit.edu/) (Rozen & Skaletsky, 1999; Koressaar, T. & Remm, M., 2007; Untergasser, A. *et al.*, 2012). They were optimised for primer length (18-25bp), product length, G/C content, annealing temperature, minimal self-compatibility and cross-compatibility and the presence of a GC clamp at the 3' end. Details for all the primers used in this research are given in the material and methods section for subsequent individual chapters.

2.5 DNA and RNA extractions and processing

2.5.1 Standard genomic DNA (gDNA) extraction

For extraction of gDNA, tissue samples were collected in liquid nitrogen and stored at -70°C until processing. Frozen tissue samples were ground using either mortar and pestle or carbide beads, and a mechanical homogeniser (Retsch MM30 or Qiagen TissueLyserII), depending on sample sizes. Samples were stabilised with 500µL of 2x Extraction Buffer (100mM Tris-HCl, 1.4M NaCl, 20mM EDTA, 2% w/v CTAB, pH 8 with HCl) and incubated for 10-15 minutes at 60°C with gentle agitation. Solvent extraction was performed twice using chloroform-isoamylic alcohol (24:1) solution. DNA was precipitated with 1mL of Precipitation Buffer (50mM Tris-HCl, 10mM EDTA, 1% w/v CTAB, pH 8 with HCl), pelleted by centrifugation for 10 minutes at 10,000g and resuspended in 300µL 1.5M NaCl containing 1µ RNase A (25mg/mL) and incubated for 10-15 minutes at 50°C. DNA was precipitated in chilled 95% ethanol, pelleted by centrifugation at 10,000g for 15 minutes, washed in 70% ethanol, air dried and dissolved in autoclaved Milli-Q water (Milli-Q Plus, Merck Millipore, Billerica, MA, USA). Dilutions of 50ng/uL gDNA were used for PCR and HRM analysis.

2.5.2 RNA extraction and cDNA synthesis

Frozen tissue samples were ground using either mortar and pestle or carbide beads, and mechanical homogeniser (Retsch MM30 or Qiagen TissueLyserII), depending on sample sizes. Total RNA was extracted using the Promega SV Total RNA Isolation System (Promega, Madison, WI) in accordance with the manufacturer's instructions. First strand cDNA was synthesised from 1µg RNA using the Tetro Reverse Transcriptase (Bioline, London, UK), or MMLV High Performance Reverse Transcriptase (Epicentre, Madison, WI, USA), each in a total volume of 20µL, in accordance with the manufacturers' instructions. To check for

contamination, a negative control without reverse transcriptase (RT-) was included for each sample. The cDNA product was diluted one in five, and used for PCR or qRT-PCR.

2.6 PCR

2.6.1 Standard PCR

Standard PCR was performed in a 50µL volume, comprising 5µL of template DNA (50 ng/µL), 10µL of 5x reaction buffer, 1µL of dNTPs (10mM), 1µL of forward primer (10µM), 1µL of reverse primer (10µM), 1.5µL $MgCl_2$ (50mM), and 0.2µL of MangoTaq™ DNA polymerase (Bioline, Alexandria, NSW, Australia), with autoclaved Milli-Q water to final volume. Reactions were conducted in a thermal cycler with heated lid as follows: 94°C for 5 minutes, 35-40x (94°C for 45 seconds, annealing temperature for 45 seconds, 72°C for 1 minute per kb according to expected product size), 72°C for 5 minutes.

2.6.2 Quantitative reverse transcription PCR (qRT-PCR)

For analysis of relative gene expression, qRT-PCR was conducted using a Rotor-Gene 3000 Real-time Thermal Cycler with Rotor-Gene 6 Version 6.1 (Corbett Research, Australia). A Corbett Robotics CAS-1200TM pipetting robot (Corbett Research, Australia) with CAS Robotics 4 Version 4.9.8 (1.6.61) software was used to prepare reactions. Each 10µl reaction comprised 2µL cDNA template, 5µL 2x Quantace SensiMixPlus SYBR reagent (Alexandria, NSW, Australia), 0.3µL each of forward and reverse primer (10µM) and 2.4µL autoclaved Milli-Q water. A no template control (containing water instead of cDNA) was included for each run to check for contamination, and each sample was run in duplicate for increased accuracy. For each cDNA sample, ACTIN was run on the reverse transcriptase negative control (RT-) to check for gDNA contamination. Reactions were run for 50 cycles. A standard curve for the target gene was included in every run. Standard curves were generated from a 10-fold dilution series from 1×10^{-1} to 10^{-6}ng/µL. Standard curve regression was considered acceptable if the R^2 value was equal to or higher than 0.99. ACTIN was chosen as the reference gene for evaluating transcript levels of flowering genes as previously described (Foo *et al.*, 2005; Hecht *et al.*, 2011); see Materials and Methods in Chapter 5 for primer details). Calculations of gene expression relative to ACTIN were based on non-equal amplification efficiencies and deviation in threshold cycle using the means for two technical replicates (Pfaffl, 2001).

2.6.3 Purification of PCR products

For sequencing, PCR products were purified using Promega Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and eluted in sterile, nuclease free water in accordance with the manufacturer's instructions.

2.7 Quantification of DNA, RNA and PCR products

Concentration of DNA, RNA and PCR products was measured with a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) in accordance with the manufacturer's instructions.

2.8 Sequence and sequence analysis

Purified DNA was sent for sequencing to Macrogen Inc. (Seoul, Korea). Sequences (.abi files) were edited manually using Geneious v8.1.8 (http://www.geneious.com; (Kearse *et al.*, 2012). To correct falsely identified bases, remove unreadable sequence at the 3' and 5' ends and group sequences in contigs. Sequence identity was confirmed by BLAST search or alignment with existing sequence and then annotated also using Geneious v8.1.8 again.

2.9 Design of molecular markers for mapping and genotyping

Whole genes or gene sections containing introns were isolated from parental lines, or mutant and wild-type, by standard PCR. PCR products were visualised and any visible size differences between the pea lines of interest were identified and used in design of size markers. If no immediate size marker was identified, PCR products were purified and sequenced and any polymorphisms found between parental lines during sequence analysis were used for molecular marker design as follows. Specific marker details are given in individual chapter materials and methods.

2.9.1 Selection of marker genes for mapping

Where possible, existing molecular markers (size or CAPS) or marker genes published in pea were utilised (e.g. (Aubert *et al.*, 2006; Bordat *et al.*, 2011). To further refine map position of mutant loci and candidate genes, close synteny between pea and *Medicago truncatula* (Choi *et al.*, 2004; Kaló *et al.*, 2004), was used to select genes as potential marker genes based on position in Medicago genome corresponding to a region of interest in pea. Other criteria for

marker gene selection included presence of multiple introns, single gene copy in Medicago as identified by BLAST search of the Medicago genome, and availability of pea sequence as determined by BLASTn search with Medicago sequence against available Pisum expressed sequence resources (generally GenBank TSAs; see relevant mapping maker tables in individual chapters for details).

2.9.2 Size markers

Size differences between pea lines that were 15bp or larger and observed through visualisation of PCR products (≥10% of PCR product size) by electrophoresis on agarose gel, were used for design of PCR-based size markers. New primers were designed closer to the deletion site if necessary to maximise relative size differences. PCR-based size markers were scored in segregating populations simply by standard PCR and visualisation of PCR products. Size differences identified through sequencing that were too small for PCR-based size markers were used in the design of HRM markers.

2.9.3 Cleaved Amplified Polymorphic sequence (CAPS) markers

Single nucleotide polymorphisms (SNPs) between pea lines that altered a restriction enzyme recognition site, and would result in visible differences in length of digest product(s) for each parental line were used for design of CAPS markers. Restriction enzyme sites were identified in sequenced PCR products with the online tool dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) and Geneious v8.1.8 (http://www.geneious.com; (Kearse *et al.*, 2012). If necessary, new primers were designed closer to restriction sites. Enzyme digests were conducted according to the manufacturer's instructions (New England Biolabs, Inc., Ipswich, MA). Prospective CAPS markers were tested on PCR products from parental lines and successful markers were used to genotype samples from the appropriate plant population(s) by standard PCR, restriction enzyme digest and visualisation of restriction enzyme products.

2.9.4 High Resolution Melt (HRM) markers

HRM markers were designed to target indels and SNPs with primers designed to amplify small fragments (<200bp). SNPs in Class 1 and 2 are easier to detect by HRM analysis and occur had a higher rate, so these are chosen in preference when designing HRM markers (Class1 and 2 SNPs are C/T or G/A and C/A or G/T respectively, which produce the biggest Tm shifts. Class

3 and 4 SNPs are C/G or A/T and produce a smaller Tm shift). HRM markers were tested and scored in segregating populations using a Rotorgene Q HRM machine (Qiagen). A Corbett Robotics CAS-1200™ pipetting robot (Corbett Research, Australia) with CAS Robotics 4 Version 4.9.8 (1.6.61) software was used to prepare reactions containing 2µL template, 0.6µL forward primer, 0.6µL reverse primer, 7.5µL HRM PCR Mix (Bioline Sensifast HRM mix) and 4.3µL sterile milli-Q water for 15µl reactions in a 100 well disc (Qiagen Rotor-Disc 100). Conditions were as follows: 95°C for 5 minutes, 50x [95°C for 10 seconds, annealing temperature (Tm; 50-60°C) for 30 seconds], 95°C for 5 minutes, 50°C for 5 minutes, HRM (temperature increasing with 0.1°C increments from 60-90°C, or from product melt temperature -5°C to +5°C). HRM results were analysed with Rotor-Gene® ScreenClust HRM® Software (Qiagen).

2.10 Linkage analysis

Classical and molecular markers were scored in plants from F_2 generation mapping populations generated from crosses between mapping parents with the F_1 generation allowed to self-fertilise. For dominant classical markers, the F_3 generation was grown to distinguish F_2 plants that were heterozygous from homozygous dominant plants, where possible. Linkage maps were constructed from estimations of genetic distance between molecular and morphological markers based on segregation data using JoinMap®4 (Van Ooijen, J.W., 2006; Kyazma B.V., Wageningen, Netherlands).

2.11 Construction of alignments and phylogenetic trees

For phylogenetic analyses, amino acid sequences of proteins or cDNA nucloetide sequences were aligned using ClustalX (Thompson *et al.*, 1997) and adjusted manually, where necessary, in Geneious v8.1.8 (http://www.geneious.com; (Kearse *et al.*, 2012). Using these alignments, distance-based methods were used in PAUP* 4.0b10 (http://paup.csit.fsu.edu/) or probabilistic methods were used in PhyML 3.2 (Guindon & Gascuel, 2003; Guindon *et al.*, 2010) for phylogenetic analyses.

2.12 Statistical analysis

All statistical analyses were conducted using a significance level of 0.05. For comparisons between two groups, two-tailed t-tests were conducted in Excel (Microsoft 2010). P-values are reported in text for each t-test.

Chapter 3

Characterisation of new loci involved in the timing of development in pea

3.1 Introduction

Most developmental responses in plants were initially characterized through anatomical and physiological studies, but it has been the use of genetics that has subsequently transformed our understanding of the underlying mechanisms. In particular, the use of mutagenesis and the study of mutants has played a significant role in generating genetic variation that reveals the existence of specific genes and their roles as part of genetic pathways involved in the control of developmental processes. As outlined in Chapter 1, early studies of vegetative phase change were complicated by its often gradual nature and by its diverse manifestations across different species. Our current understanding of vegetative phase change has come from genetic studies using mutants, either natural or induced that show changes in the timing of these developmental phases. Studies of these mutants suggest that vegetative phase change is a complex process, but indicate that there may be a conserved genetic mechanism at its heart, involving the sequential action of the microRNAs miR156 and miR172.

In legumes, despite a long history of detailed genetic work on reproductive phase change in "model" species such as pea, soybean and Medicago, there has been little work on vegetative phase change. Even at the level of morphology, it remains uncertain what features might actually constitute vegetative phase change in legumes. As in other species, changes in leaf complexity during ontogeny have been suggested to indicate vegetative phase change in pea (Wiltshire *et al.*, 1994), but this has not been systematically investigated. One important way to test this hypothesis would be to identify and characterize mutants that specifically affect the timing of these changes. Two such mutants have been initially described in pea; the *aeromaculata* (*aero*) mutants *aero1* and *aero2* (Taylor & Murfet, 2003) (Murfet & Taylor, 2004). While the most striking feature of both mutants is the strong exaggeration of the white flecking that normally occurs on leaflets and stipules of WT plants, both mutants also show accelerated leaf change and have been described as being heterochronic. In addition to the

initial *aero1-1* allele, nine other alleles are known, and those that have been characterised, show the same acceleration of leaf change as in *aero1-1*, but to varying degrees of severity (Sidorova & Uzhintseva, 1995; Taylor & Murfet, 2003). Interestingly, *aero1* also shows earlier flowering, but *aero2* does not, which shows that leaf change can be genetically uncoupled from the reproductive transition.

In addition to *aero1* and *aero2*, a number of other mutants with putative vegetative phase change phenotypes have been selected during the course of EMS mutagenesis programs. These include two mutants that show a strong promotion of leaf change superficially similar to that seen in *aero1* and *aero2*. One of these, termed *accelerated phase change 1* (*apc1*) was obtained from mutagenesis of cv. Torsdag (Weller *et al.*, 1997), and initially described in a MSc thesis by Refli (Refli, 2002). The other, termed *apc2*, was generated by mutagenesis of the Torsdag derivative dwarf line NGB5839 (Hecht *et al.*, 2007) and has not been described previously.

The aim of this chapter is to characterise the two new mutants, *apc1* and *apc2*. It presents an investigation of inheritance patterns and a detailed characterization of their phenotypes in comparison with *aero1* and *aero2*. It also includes a preliminary analysis of genetic interactions among the *aero1*, *aero2* and *apc1* mutants through a description of double and triple mutant phenotypes. Finally it describes efforts to define, map positions for all four loci, work that was undertaken as an important first step in determining the underlying molecular nature of the mutations, and to help clarify allelic relationships.

3.2 Materials and Methods

This section contains specific details of materials and methods for research included in this chapter. General materials and methods that are also relevant are described in Chapter 2.

3.2.1 Plant measurements

Details of traits measured on plants grown in this study are shown in Table 3.1. All lengths were measured to the nearest millimetre. Data from any plants that exhibited stunted or abnormal growth were excluded.

Table 3.1 Plant trait details

Number of nodes	The first two scale leaves were counted as nodes 1 and 2, with the first true leaf counted as node 3.
Internode length	Measured as the distance between the base of a node and the base of the node above. The first internode lies between the first and second scale leaf.
Total nodes (TN)	Total number of nodes with fully expanded leaves on the main stem at maturity
Node of floral initiation (NFI)	The node at which the first floral organ is produced on the main stem, regardless of whether it is fully developed or not.
Total Plant Height	Measured from the base of scale leaf 1 to the tip of the apex of the main stem.
Relative Leaf Positioning	The number of degrees from 180^{o} a leaf is borne on the axis of the plant compared to the previous leaf.
Pod Length	The distance from the top of the peduncle (where it joins the pod) to the tip of the pod (excluding the remains of the style).
Seed Number	Number of fully developed seeds produced by the plant. Aborted seeds are not counted.
Leaves Expanded	The number of leaves expanded at a given time. A fully expanded leaf has its leaflet blades completely open and will not increase further in size.
Leaf flecking	The proportion (% area) of the upper leaf surface covered by silvery-grey flecking

3.2.2 Leaf flecking measurements

Leaf flecking was measured on fully expanded leaves. Images were taken with a Canon 8800F Scanner and ArcSoft PhotoStudio 5.5 (https://en.softonic.com/s/arcsoft-photostudio-5.5). Measurements of flecking was achieved using ImageJ (Schneider *et al.*, 2012; Schindelin *et al.*, 2015).

3.2.3 Embedding and Sectioning Pea Apices (perfomed by Warwick Gill)

Shoot apices were excised from two week old pea seedlings and fixed under vacuum in 2.5% gluteraldehyde in 0.1M phosphate buffer, pH 7.2, for 12hr at 4°C. Following two buffer washes, the samples were dehydrated in an ascending acetone series in 20% increments and taken through three changes of 100% acetone. The apices were slowly infiltrated with Spurr's resin ((Spurr, 1969); ProSciTech, QLD, Australia) and polymerised blocks were hand-trimmed with a razor blade. Semi-thick sections (4-5μm) were cut with a glass knife fitted to a Reichert OmU2 ultramicrotome. The sections were expanded on a drop of distilled water on a clean glass microscope slide on a moderate hotplate and gently heat-fixed to the glass. The slides were then immersed in 0.1% (w/v) Toluidine Blue O in 1% (w/v) sodium borate solution for 30s, rinsed in distilled water, decolourised in 70% ethanol for 30s, rinsed again in distilled water and air dried. The sections were mounted in Euparal (Australian Entomological Supplies, NSW, Australia) beneath a coverslip and cured on a cool to moderate hotplate. Slides were examined with a Nikon Digital Sight DS-L1 camera (Melville, NY, USA) mounted on a Leica DM 1000 light microscope (Nussloch, Germany) with a 10x and 20x objective.

3.2.4 Primer details

The primers used on pea for mapping purposes are outlined in the tables below:

Table 3.2 Mapping Locus Details for *AERO1*

For mapping of *AERO1* and flanking genes, new molecular markers were designed to target introns of pea orthologs of genes identified using the relevant interval in the *Medicago truncatula* genome from Phytozome (www.phytozome.jgi.doe.gov). The mapping population used was *aero1* x TER. For cleaved amplified polymorphic sequence (CAPS) markers, the relevant restriction enzyme is shown in parentheses. Markers previously designed by others are referenced.

Name	Marker type	Primers	Tm (°C)	Accession number(s)	Corresponding Medicago locus (Mt4.0)	Reference
Protein of unknown function (DUF343)	HRM	TTAATTGGCCTGGAAGAGGA CATGCGAACAAGGAAGAACA	55	JI907170	Medtr5g022760	(Sussmilch *et al.*, 2015)
Ferredoxin-NADP reductase (FENR1)	CAPS (HpyCh4IV)	ATGCTTATGCCAAAAGATCCTAATGC CTCTGCTTACAGCAAAGTCAAGCCTGAAGTT	60	X12446	Medtr5g022300	(Bordat *et al.*, 2011)
CLF	HRM	CACTACATGTTCAGTGTGGCTGT TTCAATCACCGCTAACACCTC	55	PsCam056516	Medtr5g016870	This study
AERO1	Morphological	-	-			
Protein of unknown function (UNK1)	HRM	TTCTTCATTAAGCAACAAGCAA CCGAGCTAGGATCTGCAAAA	55	GAMJ01037230	Medtr5g016100	This study
Protein of unknown function (UNK8)	Size	TCTGCATTTGATGCTGATCG AATGCACCCCATAAATCTGC	57	JI922431; JR963733	Medtr5g015840	This study
AFILA (AF)	Morphological	-	-	-	Medtr5g014400	(Goldenberg, 1965; Marx, G.A., 1969)
573	HRM	TTTTTAGTTGCCTCTATAAGTAAGC CTGACATTCTTTTTGAACTGTTGA	52	GAMJ01003252 GAMJ01063424	Medtr5g012790	J. Hofer, unpublished
COTYLEDONS GREEN (I)	CAPS (EcoRV)	CAGGAAATCTTCCAAGGACTTATCC AGAGGACCCCAACATTCTACCTTG	55	AB303331; AM884277	Medtr5g011120	(Blixt, 1974; Sato *et al.*, 2007)
ZG60	HRM	TGTCAGTATCTTGCTCACTTTCTCA AACCATGTGTCTGATTCATGTGA	55	JR955394	Medtr5g008050	J. Hofer, unpublished
UNK7	HRM	CCCTTTGTGGTCTTAATGTTGC CAACTTAAGTCCCTTCTCACATGC	60	PsCam050348	Medtr5g010620	J. Hofer, unpublished

Table 3.3 Mapping Locus Details for *AERO2*

For mapping of *AERO2* and flanking genes, new molecular markers were designed to target introns of pea orthologs of genes identified using the relevant interval in the *Medicago truncatula* genome from Phytozome (www.phytozome.jgi.doe.gov). The mapping population used was *aero2* x TER. For cleaved amplified polymorphic sequence (CAPS) markers, the relevant restriction enzyme is shown in parentheses. Markers previously designed by others are referenced.

Name	Marker type	Primers	Tm (°C)	Accession number(s)	Corresponding Medicago locus (Mt4.0)	Reference
Starch branching enzyme II (SBE2)	CAPS (EcoRI)	CCCCGATGCTGATGGAAATCC CTTTGGCCCACATCAAAGCCG	60	X80010	Medtr3g115340	(Aubert *et al.*, 2006)
Aspartate amino transferase (PsAATI)	Size	CAGTTTCACAAGGAGTGTCGC CATAGCCTATGAGTTCTTAACCAG	56	GDTM01037278	Medtr3g110065	(Aubert *et al.*, 2006)
Aeromaculata 2 (AERO2)	Morphological	-	-	-	-	This study
Inner membrane import protein Tic22 (TIC22)	HRM	TCTGGCAGCTTACAAATCATC CGCTTGTTCTTCTTTTTCACC	55	JI896934	Medtr3g101630	This study
Uroporphyrinogen III synthase HemD (HEMD1)	HRM	TGTTGATGCGGTTTATAGTTGG ACACACGCAAAATGGTATTG	55	GCMH01006960	Medtr3g101350	This study
Unifoliata (UNI)	CAPS (TaqI)	CATCAGAGCTGAAAGAAGG GCTTCCTTTTCACGTTGC	55	AF010190	Medtr3g098560	(Aubert *et al.*, 2006)
Frigida-like protein (FRI)	Size	TGCAACCATTTGTTTTAAGGTC AGGGAAATTTTGGGTGGAAT	60	CT010504	Medtr3g098290	(Weller *et al.*, 2012)
Amino acid permease II (AAP2)	Size	TTTGGACCATATATGGCATATGC CAATAAAATGCAGCAATCACAGCC	55	AC141112	Medtr3g096800	(Aubert *et al.*, 2006)

Table 3.4 Mapping Locus Details for *APC1*

For mapping of *APC1* and flanking genes, new molecular markers were designed to target introns of pea orthologs of genes identified using the relevant interval in the *Medicago truncatula* genome from Phytozome (www.phytozome.jgi.doe.gov). The mapping population used was *apc1* x TER. Markers previously designed by others are referenced.

Name	Marker type	Primers	Tm (°C)	Accession number(s)	Corresponding Medicago locus (Mt4.0)	Reference
Putative methyltrasferase (PMT16)	HRM	AAGTCTCAAGTTCCATGGCC TCCAAACATGACCTTTAACTT	55	GCMF01009852	Medtr1g022255	This study
Phosphatase 2C family protein (P2CP)	Size	CCATATTCCGATCTCCAGTGC CTCTTACACCGTGAACCACC	58	GAMJ01004783	Medtr1g022030	This study
Thiol protease (ThiolP)	CAPS (MboII)	CCGAAGAGGATTACCCCTACGTGC GCTTCTCCCCAGCTACCACCCC	55	JR951316	Medtr1g018840	(Aubert *et al.*, 2006)
Transmembrane protein (TMP)	Size	CACCCACAAATCCCTCTTCC AACAGCCCATGATTTAGCGG	58	JI897402	Medtr1g017450	This study
Proteasome regulatory particle subunit 12 (PRP12)	HRM	TCTATCTGGCTGTGGTGGAC CTCGGTCATGGGGAGAAAAG	60	JR951757	Medtr1g016750	This study
Peptide/nitrate transporter (PEPTRANS)	CAPS (PciI)	GCCGTGATTCGGATCTGATGG CGGTCGTATAAAGGAATGACTAC	60	GDTM01043116	Medtr1g009200	(Aubert *et al.*, 2006)
Jumonji domain protein (JMJ)	HRM	CTAGAGTGAAGTGTAATTGTAAG TGCCAGAATAAGGAAAATGGAG	58	PsCam000030	Medtr1g008060	This study
Ribosomal protein L28 (RPL28)	HRM	GTGCTACATAGAGAATAAGAGAT AGCTAAGGTTGTTTTCTATTCT	55	JR954693	Medtr1g007480	This study
Accelerated phase change (APC)	Morphological	-	-	-	-	This study
Transmembrane ascorbate ferrireductase (TAF)	HRM	TGCTTGTTTGGTTTGCAGGT GTTACAAAACCAAATAGCCACTG	57	GAMJ01031448	Medtr1g033390	This study
Putative tonoplast intrinsic protein (PutTIP)	CAPS (HpyCH4IV)	CATGCTTTCTCACTATTTGCCGC GCAACCAAAGGTTGATGTTGAGG	57	GDJU01003590	Medtr1g077540	(Aubert *et al.*, 2006)
Cryptochrome 2B apoprotein (CRY2B)	CAPS (NsiI)	GTTCAAGCTACAAAAGTAGTGTTTAATC CATGTCCACTTTCGTCGAATA	55	GDTM01035327	Medtr1g043190	(Aubert *et al.*, 2006)

3.3 Results

3.3.1 Wild-type pea vegetative development

Changes in leaf development during ontogeny have been considered to be a clear and simple measure of vegetative phase change in several species (Moose & Sisco, 1994; Chien & Sussex, 1996; Wang *et al.*, 2011), but so far there has been little exploration of this in legumes. In order to characterize the phenotypes of putative pea mutants it is first necessary to establish a clear understanding of the "normal" wild-type pattern. Leaves on a typical wild-type pea plant show changes in morphology from the lower juvenile leaves to the upper adult leaves (Wiltshire *et al.*, 1994). The first two nodes produce only small "scale" leaves, then from node 3 onwards the plant generates true leaves, which are compound, odd-pinnate, and bear a combination of proximal leaflets and distal tendrils. At node 3 the compound leaf consists of two leaflets and a rudimentary simple, terminal tendril. Later-formed leaves develop additional leaflet and tendril pairs, to a maximum of 6 leaflets (3 pairs) and 5-7 primary tendrils (2-3 pairs plus a terminal tendril) at around the node of flowering. The number of lateral organs on a leaf will be referred to for convenience as *leaf complexity*, and its change over development of the plant will be referred to as *leaf change*.

These features are illustrated in Figure 3 for the pea cultivar NGB5839, which is the isogenic wild-type for all four mutants investigated in this study (and subsequently referred to here as WT). From leaf 3 (the first true leaf) leaves bear two leaflets, until leaf 11-12 when this increases to four leaflets, and subsequently to six at around the node of flowering (node 17-18; Figure 3.1 top). WT leaves never exceed 3 leaflet pairs, but after flowering, can revert again to two pairs. Leaves bearing an odd number of leaflets (where the distal leaflet is opposed by a tendril) can be formed in the narrow zone of transition between even-leaflet states.

This increase in complexity is also reflected in number of terminal tendrils. The first leaf (leaf 3) has a single terminal tendril at node 3, with lateral tendril pairs added in subsequent leaves to reach 2-3 pairs primary tendrils at the flowering node. Secondary tendrils are also added, but are subject to a greater degree of variation in number. As in the case of leaflet number, the number of total primary lateral organs (i.e. leaflets plus tendrils) also increases (Figure 3.1 middle) but in a more gradual rather than stepped manner. This is even more clearly seen in the highly ramified leaves of the mutant *afila* line (cv.Térèse) (Kujala, 1953), where leaflets are replaced by rachides terminating in tendrils. These results suggest that some underlying process

is driving the gradual increase in the number of leaf organ primordia. The pattern of discrete steps in leaflet number is a kind of “canalization” in which the formation of paired leaflets is a more stable state than odd-number leaflets.

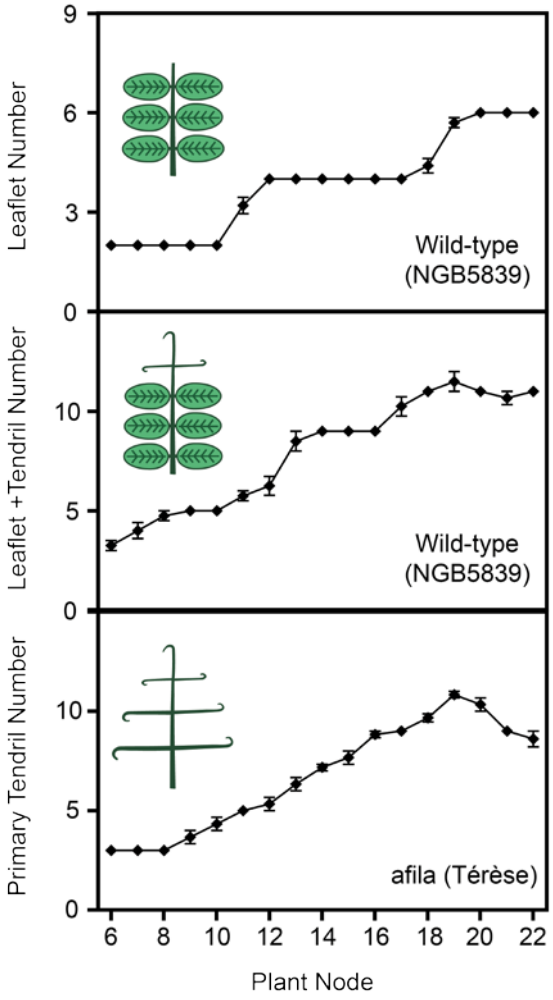


Figure 3.1 Changes in pea compound leaf structure during ontogeny

The pattern of leaflet or tendril development in wild-type (NGB5839) and *afila* (cv. Térèse) mutant plants grown under long day conditions. Diagrams are a schematic representation only. Scoring of organ number was started at node 6 as the first two nodes on a pea plant are scale leaves and nodes 3-6 were identical to node 6 and were excluded. The primary tendril number denotes each of the main branches (rachides) that terminate in a tendril(s) rather than all the tendrils visible. Values represent the mean ± standard error for 4-10 individuals per genotype.

3.3.2 Phenotypic comparisons of apc *and* aero *mutants*

In the two previously described leaf change mutants *aero1* and *aero2*, the shift to four leaflets and subsequently six leaflets is earlier (Murfet & Taylor, 2004)(Figure 3.2A; B). Also, whereas WT leaves almost never develop more than six leaflets under long day conditions, (Figure 3.6B) both *aero1* and *aero2* can produce leaves with up to eight leaflets. Initial identification of *apc1* and *apc2* mutants was also based on an earlier shift, and both mutants also typically reach a maximum of eight leaflets. To provide a comparison of the new *apc* mutants with the *aero* mutants and a more detailed picture of developmental changes in leaf complexity, all four isogenic mutants were grown together under long-day conditions.

Figure 3.2 shows that, for *aero1* and *aero2*, the transition to four leaflets and six leaflets occurs earlier than WT, but the stability of these states remains similar to WT, with a narrow transition zone of 1-2 nodes, and only rare occurrence of an "intermediate" state with an odd leaflet number. In contrast, this pattern is disrupted in the *apc1* and *apc2* mutants, where the increase in leaflet number was much more gradual, with no clearly discernible even-leaflet states. This reflected the fact that leaves with an odd leaflet number were more frequent in *apc* mutants, there was greater variability in the timing of transition between even-leaflet states, and they showed occasional reversion from higher to lower leaflet numbers during development, something that is usually only seen in WT plants in the final nodes of development as apical arrest occurs (Figure 3.2B). The tendency to exceed 6 leaflets was also stronger in the *apc* mutants than in the *aero* mutants.

In view of the fact that vegetative and reproductive phase change are known to be interconnected, it was also of interest to explore the connection between leaf change and flowering in the *apc* mutants. In WT plants, the timing of flowering usually coincides with the shift from four to six leaflet pairs at around node 17-18 in long days (Figure 3.2B; C). The *aero1* mutant was previously observed to have an early flowering phenotype, with a promotion of flowering of 1-2 nodes occurring in parallel with a similar shift in vegetative timing (Taylor & Murfet, 2003). Senescence is also 1-2 nodes earlier in *aero1* (Figure 3.2B, C). These effects of *aero1* are confirmed in the results presented in Figure 3.2C. In contrast, the *aero2* mutant was reported previously to be slightly later to flower both in terms of node of flower initiation and in time to first open flower (Murfet & Taylor, 2004). However, we saw no difference in node of floral initiation for *aero2* (Figure 3.2C), although a similar slight delay in the days to

flowering was observed (Murfet & Taylor, 2004)(results not shown). Interestingly, *apc1* and *apc2* show no change in the timing of floral initiation compared to the WT also, so that the shift to six leaflets in these mutants consistently occurs before the first floral node (Figure 3.2B; C). This further supports previous observations that although flowering and vegetative phase change can be regulated by the same genetic pathways, they can also to some extent be genetically uncoupled (Wiltshire *et al.*, 1994). It also suggests that the role of *AERO1* may be distinct in that it also affects flowering whereas the other loci appear to affect leaf change only.

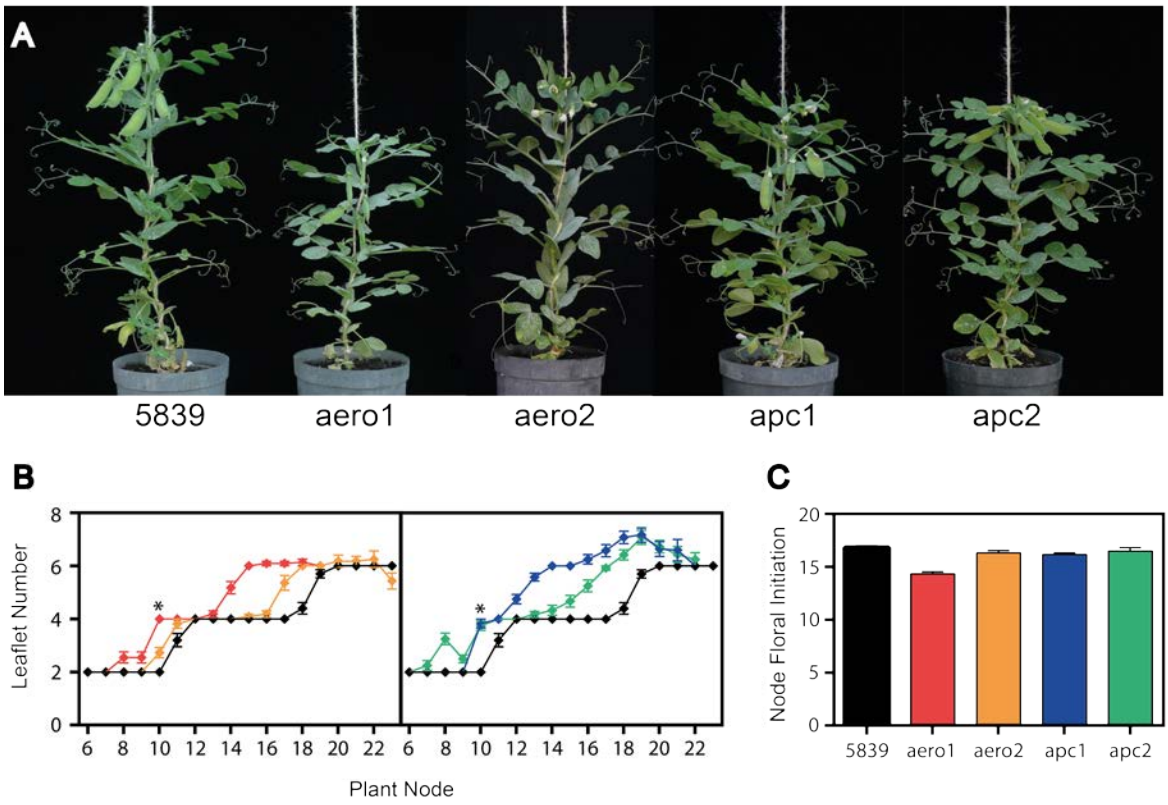


Figure 3.2 Four mutant loci that alter developmental timing

Wild-type (NGB5839), *aero1*, *aero2*, *apc1* and *apc2* plants grown under long day conditions in cabinets as per the growing conditions described in chapter 2. Photo of typical wild-type and mutant plants taken during the reproductive phase **(A)**, Total leaflet number scored at each node of plant development, starting at node 6 until last node is initiated with WT controls duplicated, **(B)** and node of flower initiation for the 5 genotypes **(C)**. Values represent the mean ± standard error for 5-6 individual plants per genotype. Star (*) denotes the first node at which both mutants were significantly different to the WT ($P<0.05$).

3.3.4 The apc *and* aero *mutants display increased leaf flecking*

During the course of genetic analyses of the *apc* mutants, it became apparent that the early leaf change phenotype was also accompanied by an increased amount of leaf flecking, but to a lesser extent than the *aero* mutants. Leaf flecking is a phenomenon that is observed across many pea cultivars and appears as silvery spots on the adaxial side of the leaf. These are due to air spaces between the epidermal and mesophyll layers that occur naturally as the leaf develops (Blixt, 1962; Marx, 1986). The flecking observed in pea could be considered as a leaf variegation phenotype, although these are predominantly caused by chlorophyll deficiencies or chloroplast abnormalities in certain areas of the leaf leading to a yellow or completely white appearance of the mesophyll (Marcotrigiano, 1997; Nair & Tomar, 2001; Sakamoto, 2003). However, another phenomenon described in the variegation literature that is much closer in nature to pea flecking is leaf blistering in *Pilea cadierei* (Vaughn & Wilson, 1981) which is caused by the collapse of the second epidermal layer increasing the intercellular space. Tomato leaf silvering may also be similar in nature (Grimbly, 1977) and is caused by a change in the cell division or expansion rate in a particular cell layer during leaf development. In pea, an extreme version of the leaf flecking phenotype may be the *argenteum* mutant, in which all leaves have a uniformly silver appearance. An early study done on this mutant (Hoch *et al.*, 1980) suggested a weakness between the epidermis and palisade call wall interface, but cell division of different layers was not investigated.

Although observed and noted in the literature for many years, leaf flecking in pea has never been quantified previously, so this study set out to measure and compare the levels of flecking for the WT cultivar and all four mutants over the course of plant development. This was done using digitally enhanced scans of a single leaflet at each node from the first true leaf (node 3) until node 12, where flecking reached its highest levels (Figure 3.3). Flecking in the wild-type cultivar, NGB5839, increases from approximately 1.7% up to a maximum of 3% of the total leaflet area (Figure 3.3). *Aero1* is strongly flecked right from the first true leaf at node three, with flecking increasing from 23% up to 95% of the total leaflet area, such that later leaves look almost completely silver in appearance. *Aero2*, *apc1* and *apc2* all start at the same low level of flecking as the wild-type, but reach a maximum flecking of 56%, 16% and 6% respectively, all significantly different to the wild-type (Figure 3.3). Leaf flecking became a useful phenotypic marker alongside leaf change to aid in identification of mutants in segregating populations detailed later.

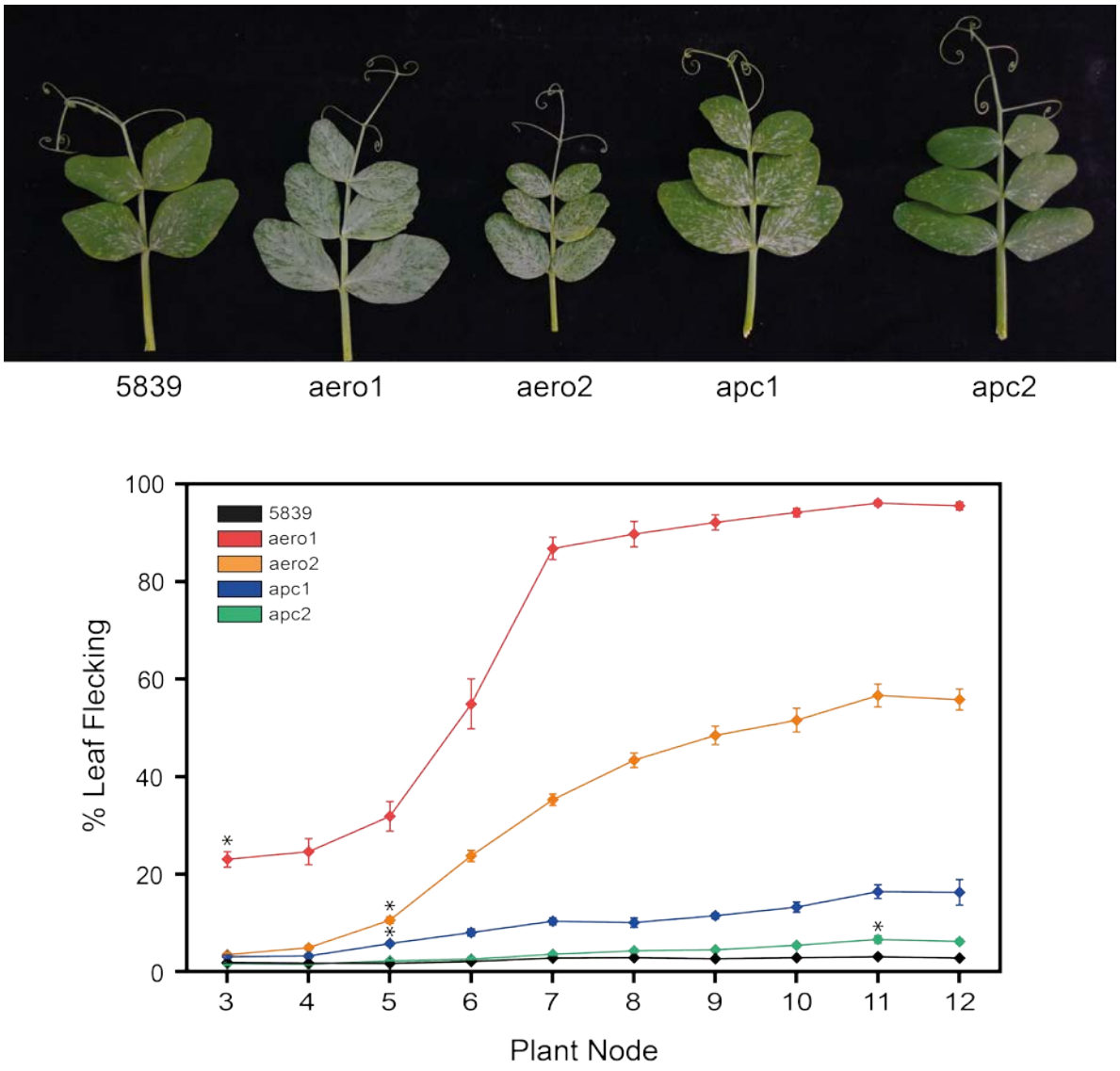


Figure 3.3 The mutant loci display increased leaf flecking

Photograph of the wild-type NGB5839 leaf flecking compared to the four mutants at node 15 and the percentage leaf flecking measured from the first true leaf (node 3) up to node 12. Plants were grown in LD conditions in the Hobart phytotron. A * denotes the first node at which each of the mutant lines differed significantly from the WT ($P<0.05$).

3.3.5 The apc1 *and* apc2 *mutants show different inheritance patterns*

Following their initial isolation, *apc1* and *apc2* mutants were backcrossed to WT (NGB5839) to examine their genetic basis and to clean up the genetic background. Like *aero1* and *aero2*, the two new mutants, *apc1* and *apc2*, accelerate and extend leaf change such that any given leaf has significantly more lateral organs than the corresponding leaf in WT (see Figure 3.2). For the purposes of examining segregation patterns I considered that the total number of leaflets produced on the plant would provide a robust indication of the mutant phenotype.

In the first part of the analysis, WT and mutant controls were grown together with the two WT x mutant F_1 progenies. Both *apc1* and *apc2* had a significantly higher total leaflet number than WT (Figure 3.4 top left), with mean total leaflet number of 83 ± 3, 75 ± 3 and 61 ± 4, respectively. This is consistent with the data shown in Figure 3.2 indicating that *apc2* has a weaker phenotype.

WT x *apc1* F_1 plants were more similar to WT than to *apc1*, indicating a basically recessive inheritance (Figure 3.4 middle left). However, they were also significantly different from WT (67 ± 2 and 61 ± 4, respectively; $P<0.05$) indicating that the WT allele is incompletely dominant with a degree of dominance of 45%. In the F_2 generation, the phenotype of the *apc1* mutant segregated in an approximate 3:1 ratio, confirming a monogenic recessive inheritance. In addition, the distribution of leaflet number in the WT class of F_2 segregants was slightly shifted towards higher values, consistent with the incomplete dominance suggested by the F_1 (Figure 3.4 top right).

In contrast, the F_1 data for *apc2* was unusual, with a much broader distribution that spanned the range between WT and the *apc2* control, and a mean total leaflet number (72 ± 4) more similar to *apc2* than to WT (Figure 3.4 bottom left). The data suggests that the inheritance of *apc2* is not recessive like *apc1*, but more likely to be partially dominant with the degree of dominance of 57%. The F_2 generation showed segregation pattern indicative of a 1:2:1 ratio, in which the majority of individuals were similar in phenotype to the mutant control, with a smaller number similar to WT or with a phenotype even more extreme than the mutant control. These data are consistent with the *apc2* mutation being essentially dominant (Figure 3.4 middle right). This difference in inheritance pattern could also suggest that *apc1* and *apc2* are fundamentally different in nature.

To test whether *apc1* and *apc2* might be alleles at the same locus, the progeny of crosses between the two mutants were examined (Figure 3.4 bottom right). Interestingly, total leaf number in the F_1 generation was variable, but overall was more similar to the mutant controls than to WT. Unlike the F_1s for the individual mutants, however, a significant proportion of individuals in the in *apc1* x *apc2* F_1 had a higher total leaf number than mutant controls, and the distribution appeared weakly bimodal. This result is in general consistent with the involvement of two non-allelic mutants – one essentially dominant (*apc2*) and the other incompletely recessive (*apc1*). An F_2 population from this cross was also grown (data not shown), and out of 66 plants, there were 3 WT individuals, supporting the previous results that *apc1* and *apc2* are non-allelic.

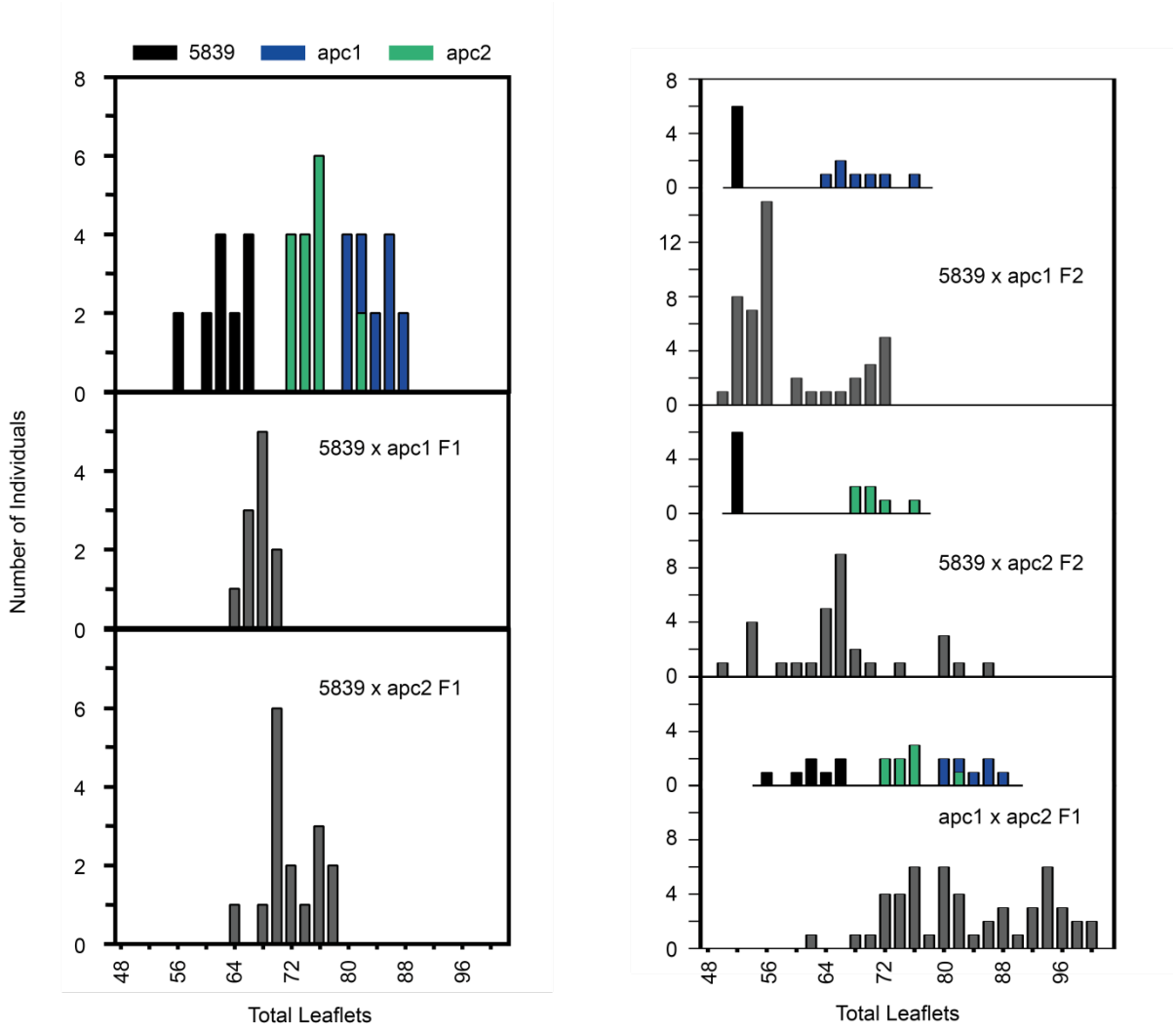


Figure 3.4 *Apc1* and *apc2* inheritance patterns

Inheritance patterns for *apc1* and *apc2* in the F_1 generation (left) and the F_2 generation (right). The F_1 phenotypic data for the *apc1* x *apc2* allelism cross is also included (bottom right). Total leaflets from node 3-20 were used for all the F_1 populations and total leaflets from node 3-18 was used for F_2 populations. The F_2 were grown at a different time and did not produce nodes above 18 consistently across the population. Plants were grown under long day conditions in the Hobart phytotron as described in Section 2.2.

3.3.6 Mapping of AERO1, AERO2 *and* APC1

Mapping of mutant loci is an important approach in an attempt to identify the underlying gene, and can also help resolve the question of allelism when there are ambiguities, such as in the case of *apc1* and *apc2* described in the previous section. In order to determine the map position of to map all four of the mutants in this study, crosses were made to cv. Térèse (TER) and F_2 progenies examined. TER has been previously used in mapping of several other mutants in the TOR/NGB5839 background (Hecht *et al.*, 2007, , 2011; Liew *et al.*, 2014; Sussmilch *et al.*, 2015; Ridge *et al.*, 2016), and is sufficiently similar to NGB5839 to have few other traits segregating, while still different enough that intronic polymorphisms can be readily identified. However, one of the difficulties with mapping leaf change mutants in this cross is that TER carries the *afila* (*af*) mutation, where the leaf phenotype has no leaflets, but has branches terminating in tendrils instead (Laucou *et al.*, 1998; Rameau *et al.*, 1997)https://www.seedstor.ac.uk/search-infoaccession.php?idPlant=26495) making leaf change/*afila* recombinants initially more difficult to recognize. However a combination of increased flecking on the stipules and a higher level of complexity in the tendrils in mutant segregants allowed their unambiguous identification, as illustrated in Figure 3.5 for *apc afila*.

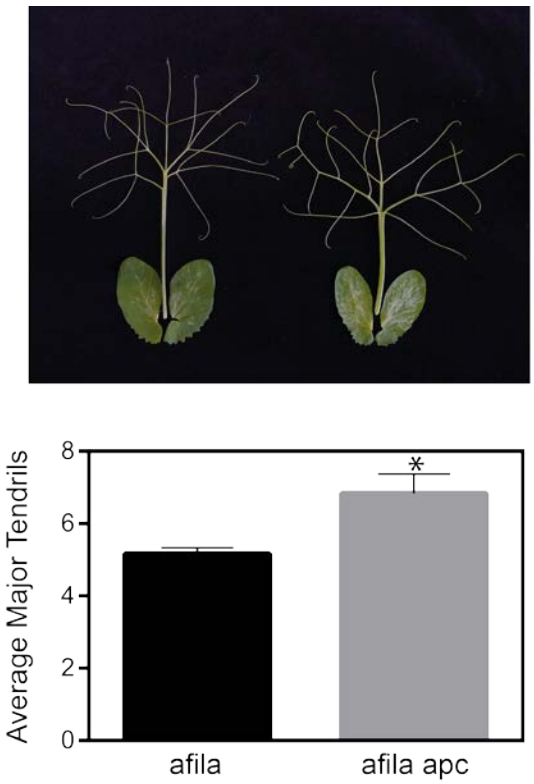


Figure 3.5 Identification of *apc1 afila* double mutant phenotype

The tendril pattern of the single mutant *afila* and the double mutant *afila apc1*. Photograph shows the stipules and tendrils of both genotypes excised from node 8. The graph depicts the average number of major tendrils, which refers to the major branches (rachides) that terminate in a tendril(s) rather than all tendrils. Plants were grown in LD conditions. Values represent the means ± standard error for 6 plants per genotype. The afila apc double mutant was significantly different from the afila single mutant (*, $P<0.05$).

Early reports suggested that the *AERO1* locus was located towards the bottom of pea linkage group (LG) I, near the classical loci *AF* and *I* (Marx, 1986). *AERO1* was mapped in the F_2 progeny and after the initial scoring, synteny between *Medicago* and pea was used to design markers in the region to confirm this position and narrow down the interval. *AERO1* now sits about 1.6cM above marker *UNK1* (Figure 3.6).

The location of *AERO2* was unknown at the start of this project. Systematic testing with published genic markers from (Aubert *et al.*, 2006) for each pea LG revealed linkage with *PsAAT1* on the top of pea LGIII. New markers across this region were then designed using the Medicago synteny and the pea/Medicago comparative map (Tayeh *et al.*, 2015), and limited *AERO2* to a 27cM interval between markers *PsAAT1* and *TIC22* (Figure 3.6). Attempts to narrow this interval further were unsuccessful due to a lack of polymorphisms for genes in this region. One explanation could be that this region contains the *EARLY FLOWERING 3* (*ELF3*) gene, which has had an important role in flowering time adaptation (Weller *et al.*, 2012), and is highly likely to have undergone a major selective sweep and a dramatic reduction in genetic diversity in domesticated varieties.

Previous preliminary efforts to map *APC1*, using classical and isozyme markers, suggested a location on LG II between the isozyme marker *AATP* and Mendel's flower colour locus *A* (Refli, 2002). This position was confirmed in the independent TER x *apc1* F_2, and after several rounds of marker design and genotyping, was refined to an interval of approximately 10cM between markers *RPL28* and *TAF* (Figure 3.6).

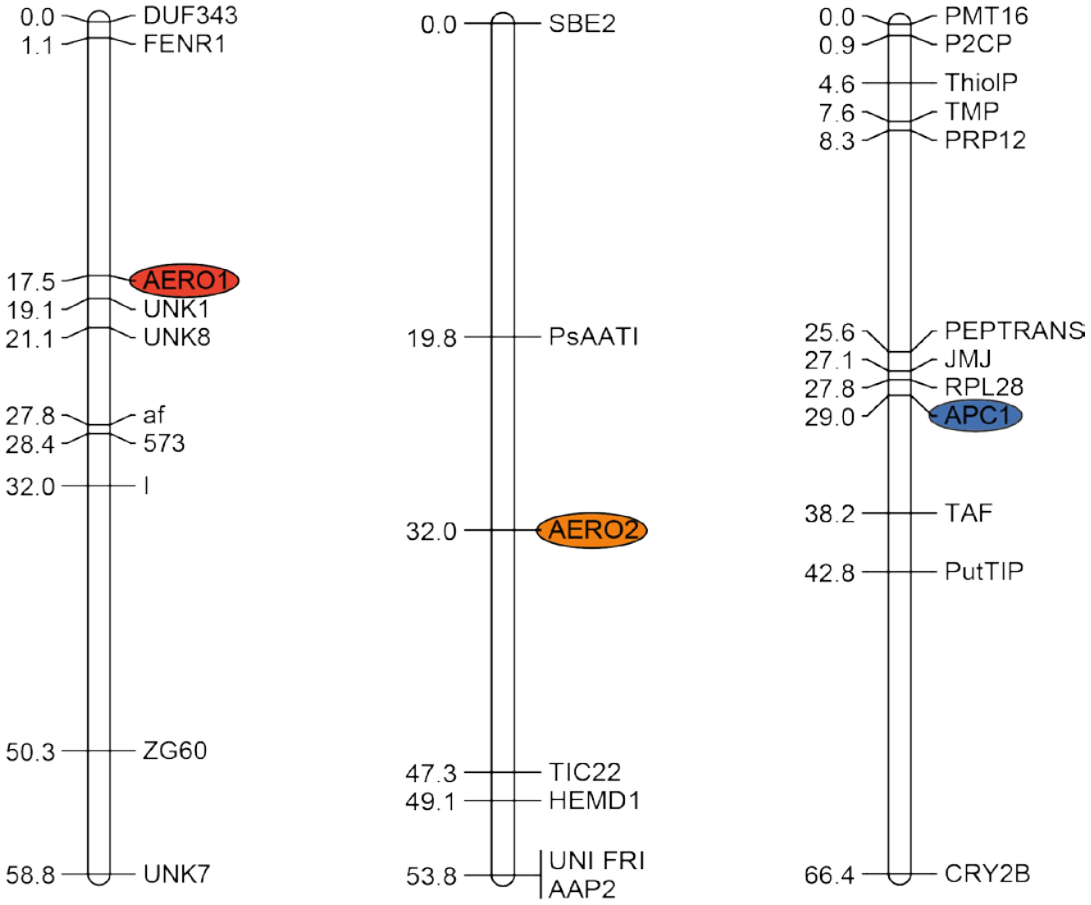


Figure 3.6 Mapping of *AERO1*, *AERO2* and *APC1* loci

Linkage maps for *AERO1*, *AERO2* and *APC1* loci were constructed from segregation data using JoinMap®4 (Van Ooijen, J.W., 2006; Kyazma B.V., Wageningen, Netherlands). All mutants were crossed with cv.Térèse to create the mapping population. *aero1* n= 60; *aero2* n=59; *apc1* initial population n=80, then increased to n=260. Marker information is outlined in Tables 3.2, 3.3 and 3.4.

Finally, in view of the somewhat ambiguous results from allelism testing with the *apc2* mutant, a mapping approach was used to examine whether *apc2* might be co-located with the *AERO2* or *APC1* loci or could represent a novel locus. Co-location with *AERO1* was not considered, as all of the *aero1* alleles that have been described have high levels of flecking on the leaves and flower earlier (Marx, 1986; Sidorova & Uzhintseva, 1995; Taylor & Murfet, 2003), and are thus distinct from *apc2*, which has no flowering defects and much lower levels of flecking on the leaves. Markers closely linked to the other mutant loci were tested on the F_2 progeny of TER x *apc2* (n=83) (Figure 3.7A), to check whether linkage was also shown with *apc2*.

To test the possibility that *apc1* might be allelic to *apc2*, the *apc2* phenotype was tested for cosegregation with the *JMJ* marker, but there was no significant linkage between *APC2* and *JMJ* (Figure 3.7A). To counter any possibility that this result might be confounded by the uncertainty in identifying the *apc2* mutant phenotype, the association of the *JMJ* marker with leaf change phenotype was also compared in the *apc1* and *apc2* mapping populations. The results show a very strong effect of *JMJ* genotype in the *apc1* population but not in the *apc2* population, confirming that *APC1* and *APC2* are two distinct loci (Figure 3.7B). A similar comparison was also made with *aero2*, using the *AERO2* flanking marker *TIC22*. In this case there was evidence of weak linkage between *APC2* and *TIC22* (Figure 3.7A), with an estimated distance of 23.8 cM between the two loci. This contrasts the strong linkage of *TIC22* with *AERO2* (Figure 3.6) and suggests that allelism with *AERO2* is unlikely. It may hint at a location for *APC2* below *TIC22*, but further work will be needed to confirm this.

A

Gene pair	P value
apc1 & JMJ	<0.00001
apc2 & JMJ	0.222
aero2 & TIC22	<0.00001
apc2 & TIC22	0.0402

B

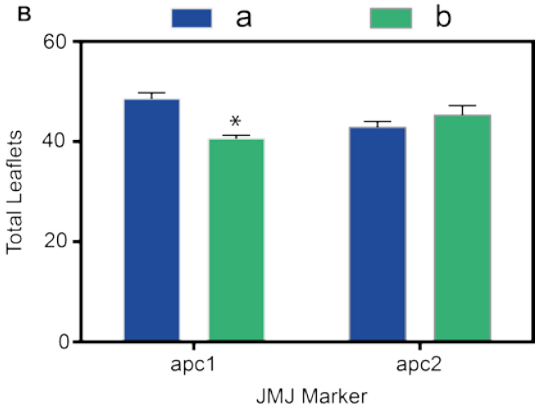


Figure 3.7 APC2 is not allelic to the other three loci.

Testing linkage significance for *APC2* with markers that are highly linked to the *APC1* and *AERO2* loci. Chi-squared tests were conducted and the P values shown (**A**). Scoring total leaflet number in the F_2 of *apc1* x TER and *apc2* x TER against the homozygous genotypes of the *JMJ* marker (**B**). Values represent the mean ± standard error of 13-17 plants per JMJ genotype. A * denotes significant difference between a and b for the JMJ marker.

3.3.7 Mutant combinations reveal additive and enhanced genetic interactions

One approach to explore whether the *aero* and *apc* mutants might have related functions is to examine their genetic interactions. Crosses were generated to enable the selection of double mutants combining *aero* and *apc* mutants, Crosses with *apc2* are not reported here in view of difficulties in unambiguously identifying homozygous mutant progeny (see section 3.3.5). For all other crosses, double mutants were readily identified in the F_2 and F_3 using a combination of phenotypic observations. Crosses between double mutants were then made in order to select *aero1 aero2 apc* triple mutants, which were again straightforward to identify based on trait combinations. During the selection process of double and triple mutants, it became apparent that as mutations were combined, effects were emerging of other traits in addition to leaflet number and flecking including floral architecture and phyllotaxy, particularly in *apc1* genotypes. This observation prompted a closer analysis of these traits in the single mutants. Table 3.5 summarizes the phenotypic features of all the genotypes.

Table 3.5 Summary of the defects exhibited in the single, double and triple mutant phenotypes. The number of diamonds indicates the severity of the defect.

	aero1	aero2	apc1	aero1 aero2	aero1 apc1	aero2 apc1	aero1 aero2 apc1
Vegetative Timing	◆	◆	◆	◆◆	◆◆	◆◆	◆◆◆
Reproductive Timing	◆			◆	◆		◆
Floral Architecture			◆		◆◆	◆◆◆	◆◆◆
Phyllotaxy	◆	◆	◆	◆◆	◆◆	◆◆	◆◆◆
Pod Architecture		◆		◆◆		◆◆◆	◆◆◆
Leaf Flecking	◆◆◆	◆◆	◆	◆◆◆◆	◆◆◆	◆◆	◆◆◆◆
Sterility						◆	◆

3.3.7.1 The timing of vegetative development

In general, as mutations were combined, the timing of leaf change became earlier and the total number of leaflets increased. Whereas all single mutants exhibited six leaflets at node 15, the double mutants had seven or eight leaflets, and the triple mutant had nine or ten (Figure 3.8A, 3.9E). This suggests that the genes defined by these mutantions converge on the same fundamental processes that control leaf patterning and its regulation during development.

Another example of the additive effect of additional mutations on the timing of development was seen in the rate at which new phytomers (nodes with associated leaf) are sequentially added over time. This can be expressed as the plastochron index which represents the time taken for each phytomer to emerge. There was no difference in rate of phytomer development among any of the genotypes at two weeks after sowing, but differences began to appear later in development. By six weeks after sowing the plastochron index was 7-10% higher than WT for the single mutants, 9-13% higher than WT for the doubles and 21% higher for the triple mutant. (Figure 3.8A) This lower rate of development did not affect the total number of nodes the mutant plants produced. In fact, all mutant combinations produced a greater number of nodes overall, except in the case of *aero1*, in which flowering is earlier (Figure 3.8D). Interestingly, even though more nodes are produced, the overall plant height was still significantly reduced in all the mutants, due to a reduction in internode length, as previously described for *aero2* (Murfet & Taylor, 2004). (Figure 3.8A, 3.8D).

3.3.7.2 Flower and pod development

Other phenotypes that became conspicuous in the double and triple mutants involved defects in floral and pod architecture. Apart from the *aero2* mutant which was previously described to affect pod morphology (Murfet & Taylor, 2004), none of the single mutants were initially observed to have any obvious effects on reproductive development. However, a close examination showed that the *apc1* single mutant occasionally exhibits abnormal development of flowers and inflorescences, including the formation of smaller flowers that do not always completely open. The *apc1* mutant also sometimes fails to complete secondary inflorescence development, with the terminal stub appearing to convert to a bract or an abnormal floral structure (Figure 3.8C). Even though such flower/inflorescence abnormalities are not seen in *aero1* or *aero2* single mutants, when these mutations are added to *apc1* the floral abnormalities are consistently enhanced, suggesting that the *AERO1* and *AERO2* genes may act in a partially

redundant manner with *APC1* to control the development of reproductive structures (Table 3.4; Figure 3.8C).

A similar enhancement of mutant phenotypes is also seen for pod defects. Consistent with previous observations of (Murfet & Taylor, 2004), pods of the *aero2* single mutant were significantly shorter and wider than WT, and produced only half the number of seeds per pod. Neither the *aero1* or *apc1* single mutants exhibited pod defects (Figure 3.8D, 3.9C), but the addition of these mutations to *aero2* resulted in a striking effect. The *aero2 apc1* double mutant and the *aero1 aero2 apc1* triple mutants are completely sterile, and produced only occasional very small deformed pods that never contained viable seed. During the first few nodes of flowering, often small floral buds are produced that do not develop into full flowers and frequently abort. Later, smaller flowers are formed and are apparently normal in structure, but they do not open properly.

The *aero1 aero2* double mutant has even shorter wider pods than the *aero2* single mutant, but had no additional effect on seed number relative to aero2 (Figure 3.8D; Figure 3.9C). Finally, even though there are no pod defects in the *aero1* and *apc1* single mutants, pods of the *aero1 apc1* double mutant appeared similar to those of *aero2*, and like *aero2*, produced fewer seeds, with only one seed per pod on average (approx 33% relative to WT and single *aero1* and *apc1* mutants). These results again suggest there is a likely redundancy in the effects of these genes in control of pod and seed development.

3.3.7.3 Phyllotaxy

A further striking feature emergent in the double and triple mutants was perturbed phyllotaxy, which was seen most clearly at the later stage of development. In pea, leaves are normally formed in a distichous arrangement. Thus, as the plant develops and adds additional nodes, the youngest leaf emerging is positioned at approximately 180 degrees from the previous one on the stem axis. In WT plants there is some variation around this value such that the angle may differ by up to 10^{o} from 180^{o} but successive leaves compensate in the opposite direction so a distichous arrangement is maintained overall. However, in all mutant combinations this relative leaf positioning is altered so that the angle difference from 180^{o} is consistently higher, leading to a more radial arrangement when viewed from above (Figure 3.9B).

A distinct but possibly related phenotype seen in *apc1*, and any mutant combinations homozygous for *apc1*, is a tendency for multiple flowers and leaves to arise out of the same node rather than a single leaf and flower, as in WT (Figure 3.8B). This is reminiscent of a similar phenotype seen in pea mutants showing stem fasciation (Marx & Hagedorn, 1962; Sinjushin & Gostimskii, 2008; Krusell *et al.*, 2011), which results from defects in the control of cell division within the apical meristem and hence in meristem organisation. These results suggest that the apical meristem control of phyllotaxy and organ positioning might be disrupted in the mutants containing *apc1*.

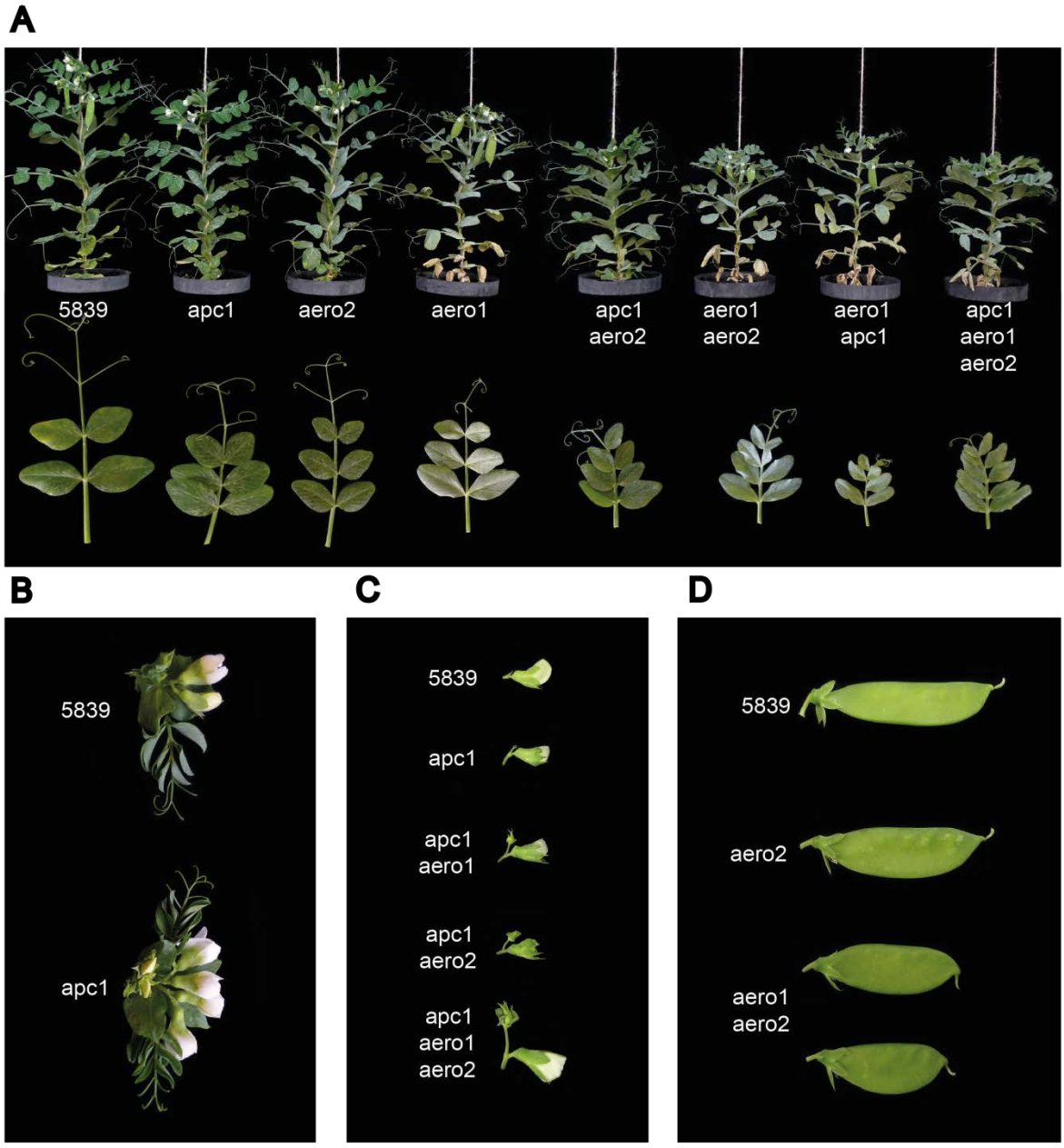


Figure 3.8 Double and triple mutant phenotypes reveal additive and enhanced interactions

Seven week old single, double and triple mutant plants photographed at the reproductive stage with a representative leaflet shown for each genotype below at node 15 (**A**). Example of perturbed nodal arrangement in the mutant *apc1* compared to the wild-type NBG5839 (**B**). Examples of floral defects in mutants containing *apc1* (**C**). Examples of pod defects in *aero2* and *aero2 aero1* mutant combinations (**D**). All plants were grown in LD in the Hobart phytotron.

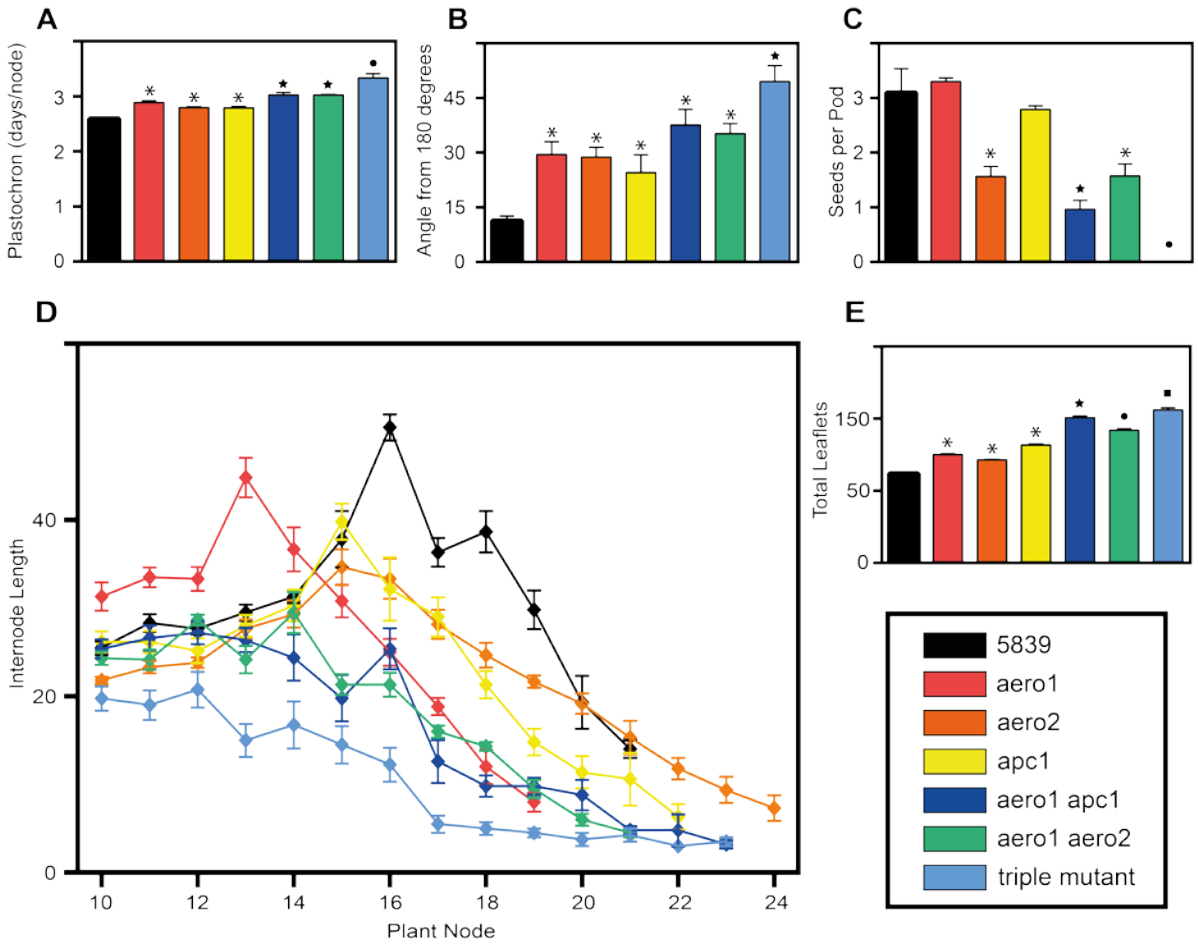


Figure 3.9 Additional mutations show additive or enhanced defects in leaf expansion, phyllotaxy, seed production and internode length.

Plastochron rate calculated at 6 weeks of age (**A**), relative leaf positioning - the leaf angle (difference from 180º) in relation to the previous leaf for the top ten nodes of plant growth (**B**), the average number of seeds per pod (**C**) and the length of the internodes from node 10-24 (**D**). Values represent the mean ± standard error for four to eight plants per genotype. The genotypes are outlined in the legend (bottom right), with black representing the wild-type NGB5839, the single mutants in warmer colours and the double and triple mutants in cooler colours. Note that the double mutant data for *aero2 apc1* is not included here as *aero2* families segregating *apc1* were used. Since genotyping of mutants was unavailable, the families all proved to be WT for *apc1* and no double mutant segregants were available for scoring in this sowing. Each symbol denotes any genotype that is significantly different from the others ($P<0.05$).

3.3.8 Mutants reveal perturbed apex and leaf primordia development

The mutants studied affect multiple plant development processes including compound leaf formation, organ positioning and reproductive development. These phenotypes are likely to reflect differences that arise very early in the process of organ initiation and development. New organs are formed on the lateral boundaries of the apical meristem in a spatially ordered and sequential manner through the process of cell differentiation, division and expansion.

In order to examine whether any of the mutants might show obvious defects in morphology or cellular structure of the developing shoot apex, longitudinal sections through the centre of shoot apices from two-week-old WT and mutant plants were compared. At this age, there are no differences in rate of node development between the mutants and WT as described previously in Section 3.3.7.1. The youngest leaf primordium, seen as a tiny protrusion from the apical meristem, is leaf 14. This captures the shoot apex at a time when primordia 15 and 16 are being initiated and thus the point at which the mutants are changing to 6 leaflets, but the WT is not. The results presented earlier in Figure 3.2 show that at node 10, the mutants have all changed to four leaflets, but WT has only two leaflets, and consistent with this, leaf change was clearly visible in leaf primordium 10 for all the mutants in multiple sections. A representative section from the *aero1* mutant is shown in Figure 3.10 as an example. Leaf primordium 10 in *aero1* has three domains visible, representing the two leaflet pairs and the terminal tendril, whereas the WT has only two domains, corresponding to a single pair of leaflets and the terminal tendril.

It was therefore of interest to see whether any differences in the cellular structure of the apex in the mutants could be detected. There was no significant difference in the number of cells in the apical meristem itself, except for *apc2*, which showed a slight increase (Figure 3.10). However, there were observable differences in the shape of the apex for the mutants, with *aero1* & *aero2* showing a more axially compressed apex and *apc1* showing a slightly elongated apex as represented by the measurements for N2-N3 and also visible in the picture for *aero1*. Although, there were differences in the shape of the apex, they were not consistent across all four mutants. However, in view of the exaggerated phenotypes seen in double and triple mutants, it would be of further interest to look at sections of these genotypes also, where any differences in meristem structure might be more apparent. A series of sections at different ages would also provide useful information for exactly when during primordia development these changes occur.

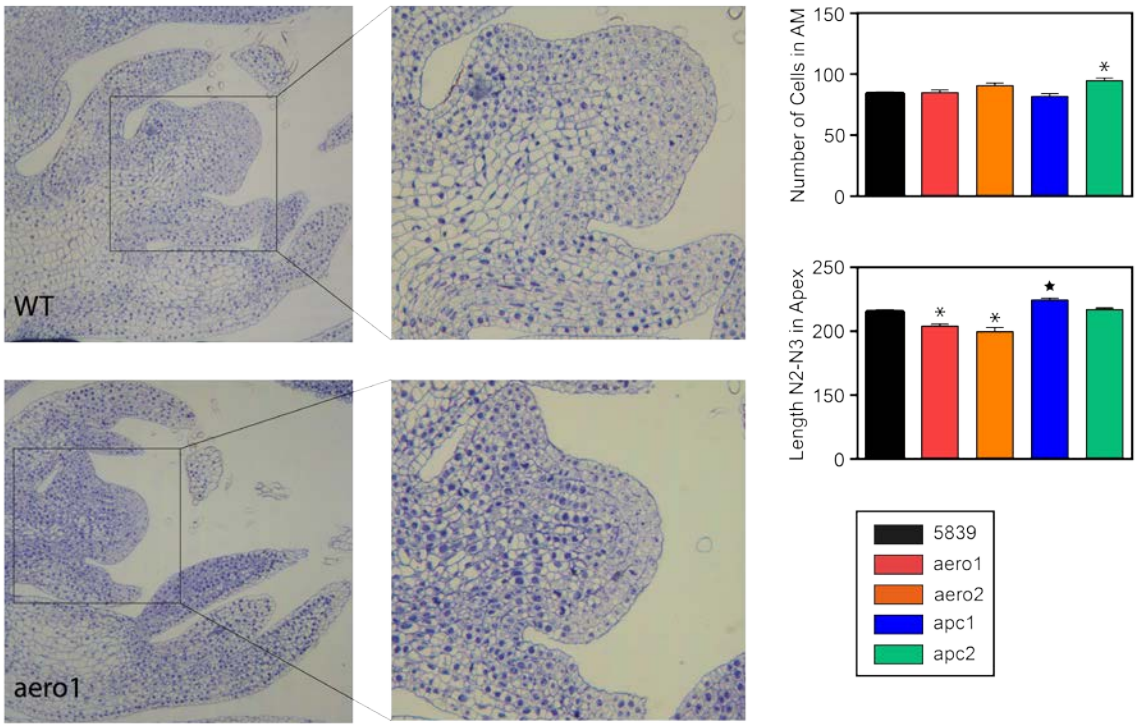
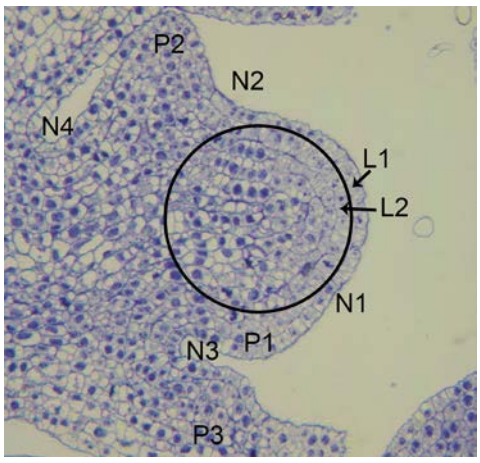


Figure 3.10 Aero1 exhibits perturbed apex and leaf primordia development

Light microscope images of apex sections of wild-type NBG5839 and *aero1*. On the left is the 10x image and the black box indicates the area on the right that is enhanced to 20x to show the cells in apical meristem and the youngest leaf primordia developing. Plants were two weeks old and the youngest leaf primordia bulge (P1) is node 14. There is no difference in the rate of leaf development at this young stage between the genotypes. Values represent the mean ± standard error for 4 plants per genotype and a single section per plant. Any significant differences are highlighted with a symbol ($P<0.05$). The number of cells in the apical meristem (AM) was determined using a 200µm diameter circle with the edge of the circle aligned to the boundary between the L1 and L2 cell layers. The length of the N2-N3 was determined by measuring the distance (µm) between top of leaf primordia 2 (P2) to the top of leaf primordia 3 (P3). The legend (bottom right) indicates the genotypes used. Materials and methods for embedding and sectioning are described in Section 3.2.4. Diagram below shows different parts of the apex labelled:



3.4 Discussion

Vegetative phase change has been a subject of significant interest over the last two decades and attempts have been made to define this process and understand its genetic control in a number of species. With only limited previous attention given to vegetative phase change in pea, we wanted to explore this process further, with particular interest in the question of whether morphological changes in compound leaf development during ontogeny are indeed a manifestation of phase change.

Observations of wild-type pea development have shown that the compound leaf increases in complexity with age, through the addition of more lateral organs (which may be tendrils or leaflets) on the main leaf axis. Consideration of leaflet number alone gives the impression that the process is tightly staged, with distinct, short, intermediate transition periods between stable states with two, four or six leaflets. As the change to six leaflets occurs around the node of flowering in WT, we could hypothesise that since this coincides with reproductive phase change, the earlier shift from two to four leaflets may be indicative of the juvenile to adult vegetative phase change. However, when leaf complexity is represented by the total number of lateral organs, there is a more gradual progression, which is particularly clear in the tendril only mutant, *afila* (Kujala, 1953). This calls into question the idea that the discrete changes in complexity represent distinct transitions in progress from a juvenile to an adult state, and suggests that they represent an underlying continuous gradient of growth. These observations also suggest that the process of leaflet formation is more complex than a tendril, requiring a specific developmental decision with a higher threshold for initiation. This could be linked to the fact that a leaflet is a more complex organ, therefore presumably requiring a higher allocation of resources. One way to approach this problem in future might be to look at whether there is any change in leaflet morphology (such as an increase in size or complexity of venation) over a series of leaves in the two or four leaflet stage, which may provide an indicator of the underlying developmental gradient even though the number of leaflets is not changing.

Study of phase change in plants such as Eucalyptus and ivy (Robbins, 1957; James & Bell, 2001), that show dramatic transitions between a juvenile and adult leaf form, has established the idea that juvenile and adult stages exist as discrete and definable. However, the observations presented in this chapter suggest that in the pea system, discrete changes in phase cannot be clearly defined, at least through obvious features of leaf development. Therefore, a clear

distinction between juvenile vegetative and adult vegetative stages in pea may not be possible due to the gradual and continuous nature of vegetative development. If these two stages exist, it seems more likely that the transition occurs over a number of nodes and is not specifically related to the time the pea plant shifts from two to four leaflets, as this is merely a consequence of the resource requirement and/or developmental threshold for leaflet formation compared to tendril formation.

Most previous work on vegetative phase change has been conducted in Arabidopsis. Leaf shape in Arabidopsis does vary during shoot development, but the variation is gradual and it was difficult to identify factors that affect vegetative phase change simply by their effects on leaf complexity alone, similar to the results found in pea. (Medford *et al.*, 1992; Telfer & Poethig, 1994). However, it was subsequently shown that the distribution of trichomes on leaves in Arabidopsis can be used in combination with leaf shape and size to monitor phase change (Lièvre *et al.*, 2016). Leaves produced during early rosette development are smaller, rounder and lack trichomes on their abaxial surfaces. Leaves produced in the adult stage are larger and more elongated with trichomes on both surfaces, and leaves in the inflorescence are smaller bracts that have few or no trichomes on their adaxial surfaces (Telfer & Poethig, 1994; Chien & Sussex, 1996; Lièvre *et al.*, 2016). Multiple trait combinations are also used in maize to distinguish the juvenile and adult phases of development, including leaf shape, internode distance and epidermis characteristics (Moose & Sisco, 1994; Bongard-Pierce *et al.*, 1996). Very recently it was shown that different types of trichomes in tomato are a clear marker for the juvenile and adult phases of vegetative development (Vendemiatti *et al.*, 2017). In the pea system, there may also be other specific traits or a combination of traits, such as stem structure, epidermal traits, leaf venation or shoot apical meristem size that may prove more useful in future to help clearly define vegetative phase change in pea.

Nevertheless, even if vegetative phase change cannot be specifically defined by leaf change alone in pea, additional evidence to suggest leaf change is associated with developmental timing is found in the four mutants investigated in this chapter, *aero1*, *aero2*, *apc1* and *apc2*, that all shift the timing of this trait during plant development. All four mutants promoted leaf change by at least two nodes compared to the WT isogenic line. If the timing of compound leaf development is accelerated, then this may be related to a reduction in the length of the juvenile phase. This is most likely to be the case in *aero1*, as this mutant also exhibits a promotion of flowering and senescence suggesting that *AERO1* acts to delay the entire ageing process and is

a master regulator of developmental timing (Taylor & Murfet, 2003). However, the other three mutants do not accelerate flowering or senescence. Thus, leaf change and flowering time can be separately controlled in pea, and *AERO2*, *APC1* and *APC2* may function only in the earlier process.

There are a number of mutants in Arabidopsis that exhibit changes in the timing of leaf development, which are also associated with the timing of vegetative phase change. The *paused* (*psd)* mutant delays leaf production, but has fewer leaves without adaxial trichomes, an indicator of an acceleration of the juvenile phase in Arabidopsis (Telfer *et al.*, 1997). This mutant appears most similar to the pea mutants, which also show a slight delay in the rate of leaf production, but an acceleration of leaf change. In addition, the vegetative phase change mutants in Arabidopsis also vary in whether they also effect the timing of flowering. The *serrate* mutant has defects in both the juvenile vegetative and adult vegetative phase duration, but not the timing of flowering (Clarke *et al.*, 1999). Other mutants, such as *hasty*, show both a reduction in length of the juvenile phase and an acceleration in the timing of flowering, similar to the pea *aero1* mutant (Telfer & Poethig, 1998).

However, the idea that the pea mutants affect components of the phase change pathway affecting leaf complexity, does not take into account the other defects associated with these mutants, such as increased leaf flecking, fasciation, impaired floral and pod development, perturbed phyllotaxy and reduced plant height. One possible explanation is that the *AERO* and *APC* loci are in fact general regulators of plant development that are involved in essential cellular functions, rather than specific to phase change. They may be affecting phase change by regulating components of the conserved pathway, but may also regulating other major growth and development pathways, including compound leaf formation, flower and pod formation and organ positioning. These broad-scale regulators could include proteins that have general housekeeping (e.g. transport and biogenesis of key cellular components) or regulatory functions (e.g. in protein degradation, transcription or epigenetic modification). Known mutants in these types of proteins often display pleiotropic defects, as observed in *aero* and *apc* mutants. Interestingly, the Arabidopsis *paused* mutant mentioned above has multiple defects and was initially described as a regulator of phase transitions by (Telfer *et al.*, 1997). It was only later that it was identified as having a mutation in an exportin-t homolog, a transporter protein that mediates nuclear export for tRNAs (Hunter, 2003; Li & Chen, 2003). Another Arabidopsis example is the *serrate* mutant, that was also originally defined as having phase

length defects (Clarke *et al.*, 1999) and later shown to encode a zinc-finger protein involved in microRNA biogenesis (Yang *et al.*, 2006; Laubinger *et al.*, 2008).

A further indication that the four pea loci are governing multiple aspects of development, consistent with a possible role as general regulators, is provided by the nature of their interactions. In general, as mutants were combined in double and triple mutants, the severity of the phenotypes and the range of traits affected increased, suggesting the possible involvement in multiple pathways regulating the same downstream processes. For some traits, the severity of the double mutant phenotype was enhanced, meaning it was much stronger than the expected summation of the single mutant phenotypes. For example, *aero2* enhanced the floral defects seen in *apc1*, even though there were no floral defects in the *aero2* single mutant. The same enhancement of pod deformity was seen when the *aero1* mutation was added to *aero2*. One possibility to explain this is if two major regulatory proteins are disrupted. Even though they may not be related, this can significantly increase the impact on the normal functioning of the plant and cause greater deleterious consequences that may not be seen if only a single regulatory protein is disrupted. This is likely to be the case in the *aero2 apc1* double mutant, where the single mutants are viable on their own, but when combined, the *aero2 apc1* plants become completely sterile.

Another possibility for enhanced genetic effects can also be due to homologous proteins functioning either in a partially or completely redundant manner, so that in the single mutants the other loci is sufficient enough for proper functioning, but only when both are knocked out is the function impaired. There are many examples of this in the literature, but a striking one is the two related protein phosphatases essential for stem cell maintenance and specification in Arabidopsis, POLTERGEIST (POL) and POLTERGEIST-LIKE 1 (PLL1). Both single mutants display little or no phenotype (Yu *et al.*, 2003; Song & Clark, 2005). However, when the *pol pll1* double mutant is seedling lethal due to severe defects in the regulation of cell proliferation in the meristem, showing that POL and PLL1 clearly operate redundantly. Among the three mutants combined in this study, *apc1* and *apc2* have the most similar phenotypes, and could be most likely to functioning redundantly. Although the double mutant was too difficult to identify in the F_2 due to the partially dominant nature of *apc2*, the F_1 generation gives some clues. Some of the heterozygotes had a more severe leaf change phenotype than either of the single mutants and these individuals could represent the double heterozygotes. This type of

interaction is termed non-allelic non-complementation and suggests that these two proteins could function interactively in some type of complex to control leaf change.

Although many possible conclusions can be made about general nature of the loci we are investigating, there will be few specific answers available until the molecular identity of these loci are uncovered. The question remains, are these genes involved in the known phase change pathway or components that regulate it as outlined in chapter 1, or something else entirely? Further fine mapping and candidate gene identification will be the focus of the following chapter.

Chapter 4

Molecular characterisation of *AERO1* and *APC1*

4.1 Introduction

Forward genetics approaches have been vital in the discovery of genes involved in various developmental processes, including vegetative phase change. While the initial isolation and characterization of mutants is relatively straightforward, involving mutagenesis, screening and genetic analysis, subsequent molecular identification of the underlying genes is often more challenging. In many cases, mutant phenotypes are observed long before any idea of which gene is responsible. The degree of difficulty in conclusively identifying the causal gene for a specific genetic locus depends on a number of factors, including the type of mutagen used to generate the mutation, the size and complexity of the genome, the availability of reference genome and transcriptome sequences, and the ease with which the organism can be transformed.

In some model plant systems, gene tagging approaches can provide a simple way to determine the molecular nature of the mutation, but this approach is not available in pea. Most induced mutants in pea have been generated by chemical or radiation mutagenesis and their molecular characterization has involved a combination of positional and candidate approaches. This involves mapping loci to a varying degree of resolution and investigating possible co-location with genes known from other systems to have some functional similarity to the locus under study. Uncovering the molecular nature of the Mendelian loci are examples of this approach (Bhattacharyya *et al.*, 1990; Hellens *et al.*, 2010; Lester *et al.*, 1997; Martin *et al.*, 1997; Sato *et al.*, 2007) and our research group has had significant success using this approach in recent history (Hecht *et al.*, 2007; Liew *et al.*, 2014; Sussmilch *et al.*, 2015; Ridge *et al.*, 2016).

The genome sequence is also not available in pea, but a high degree of synteny with other legumes such as Medicago and chickpea have allowed gene order and content in pea to be inferred with a high degree of reliability (Tayeh *et al.*, 2015). In combination with the availability of a high quality reference transcriptome and the increasing accessibility of next generation sequencing, this has also opened up the prospect of a purely positional approach in which coding sequences in a particular genomic region can be scanned for presence of mutations.

The previous chapter described the characterisation of two putative vegetative phase change mutants, *apc1* and *apc2*, in parallel with a more detailed analysis of previously described *aero1* and *aero2* mutants. The most interesting common feature, and the most relevant to this study, was the leaf change phenotype, with all four mutants showing a significant advance in progression through the normal series of increases in compound leaf complexity. Additional pleiotropic effects in leaf arrangement and reproductive organ development also seem to be common to all mutants as a group of traits that are initiated in the apex of the plant. While it is not clear how these combined sets of traits in the mutants might relate to phase change pathways, the most important next step in understanding their function and how they work together is to identify their molecular nature.

This chapter focuses on attempts to identify the causal genes for two mutants, *aero1* and *apc1*, for which the genetic inheritance is clear and map positions are well defined. This work involved scanning of the respective genomic regions for potential candidates, the generation of candidate gene markers for fine mapping and testing co-segregation of the mutant loci, and through sequencing. Phylogenetic analysis of specific candidate genes was also undertaken to better understand their history and conservation in pea and other legumes.

4.2 Materials and Methods

This section contains specific details of materials and methods for research included in this chapter. General materials and methods that are also relevant are described in Chapter 2.

4.2.1 RNA Sequencing

Construction of cDNA libraries and sequencing (performed by Valérie Hecht)

Harvested tissue used in RNAseq experiment consisted of dissected embryos of 2 days imbibed seeds (embryo), both leaflets from leaf 7 (leaf) and dissected apical buds (~2mm) (apex) from two plants grown in constant light for 2 weeks. Samples were frozen in liquid nitrogen and total RNA extracted using the SV Total RNA Isolation System (Promega). RNA concentration and RNA quality were determined using a Fragment Analyser (Advanced Analytical). Sequencing library preparations were conducted with 1µg of total RNA (1/3 Embryo RNA, 1/3 Leaf RNA, 1/3 Apex RNA) using the Truseq Stranded Total RNA library preparation kit with Ribozero Plant (Illumina). WT and mutant libraries were sequenced together on a Miseq sequencer using Miseq Reagent v3 150 cycles kit (Illumina). Quality check of sequenced reads was performed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

SNP detection from RNAseq data for APC1

The interval region at the top of linkage group 2 for *APC1* was determined from the previous mapping data as shown in chapter 2. The reference sequence was created using the PsCam accessions (Alves-Carvalho *et al.*, 2015) in the *APC1* interval region, aligned and matched from the Medicago genome, in Galaxy (https://usegalaxy.org/; Afgan *et al.*, 2016). Reads from the wild-type (NGB5839) and mutant (*apc1*) were paired and mapped against the reference sequences in Geneious v8.1.8 (http://www.geneious.com; (Kearse *et al.*, 2012). The wild-type and *apc1* sequences for were then aligned and checked for SNPs.

4.2.2 Primer details

The following is a table of primers used for sequencing *aero1*, *apc1* and *apc2* for SNP detection. The full *CLF* cDNA, except for approximately 30bp at the 3' and 5' ends, was sequenced in both the wild-type NGB5839 and *aero1* mutant to determine whether a SNP was present. For *apc1*, the full length *NUP85* cDNA was sequenced, then the short section around the putative SNP discovered through RNA-seq data for *FtsH* was sequenced. For *apc2*, the full *FtsH* cDNA was sequenced, except for 50bp at the 3' end.

Table 4.1 Primer details for SNP detection in *aero1*, *apc1* and *apc2*.

Primer	Mutant	Sequence (5' to 3')	Tm (°C)	Purpose
PsCLF-5F	*aero1*	CGGCGGAAAGAAATAAATTG	58	Full length CDS
PsCLF-3R		GGAAGCCGAAGTAGCATCC	58	
PsCLF-4F		CAGACTGATGTTGAAGACAAATCC	58	
PsCLF-4R		GCCACTATGAACAAAACAACTCC	58	
PsNUP85-6F	*apc1*	CAAAACCCTAAAACACACTCCC	58	Full length CDS
PsNUP85-6R		CAAGTTTCTTAAGCCCTCACG	58	
PsNUP85-7F		CAGCTGTTGGAAATTTAGGAG	58	
PsNUP85-7R		TGGCATTGCTATTCATCCATCC	58	
PsFTSH-1F	*apc1*	TCCAGCTAAATTTACGCGCC	59	SNP confirmation
PsFTSH-1R		TGCAATGTCTTCTTGGTGTGG	59	
PsFTSH-10F	*apc2*	AAAATCTCAAGCCGGCGC	59	Full length CDS
PsFTSH-10R		CTGAAGAACCAATCCCACCC	59	
PsFTSH-11F		CACGTTTTGTATCAGAGGTTGTC	59	
PsFTSH-11R		TGTGAATTGGATGTGCACCG	59	
PsFTSH-12F		AACCTGGTGAACATTGCTGC	59	
PsFTSH-13R		TTCTGGCAGTCTTCCTTCCC	59	

4.2.3 Sequence details for alignments and phylogenetic analysis of CLF

Identification of CLF and related E(z) family members were identified in BLASTp searches of species databases (see online resources in Chapter 2; Table 4.2). Using the predicted protein sequences for AtCLF, AtSWN and AtMEA, *Medicago* coding sequences identified and were then used to tBLASTn to the PsCameor database (Alves-Carvalho *et al.*, 2015; http://bios.dijon.inra.fr/FATAL/cgi/PsUniLowCopy.cgi) to retrieve homologous sequences. E(z) family members were also identified in other legume species using the Medicago coding sequences with tBLASTn searches in genome resources for soybean, chickpea and common bean. When legumes CLF sequences were aligned, there were some cases where identified genes were not annotated or where alignments were incorrectly annotated. In these instances protein sequences were inferred from transcript sequence based on alignments between species. Most instances of incorrect annotation involved short sequence duplications, incorrect intron/exon boundary locations or short sections missing from the start/end of the gene.

Table 4.2 Sequence details for CLF and E(z) related family proteins used for alignments and phylogenetic analysis

Species	Gene Name	Accession	Locus Number	Reference(s)
Arabidopsis thaliana	AtCLF	NP_179919	AT2G23380	(Springer *et al.*, 2002)
	AtSWN	NP_56722	AT4G02020	
	AtMEA	NP_563658	AT1G02580	
Brachypodium distachyon	BdCLF	XP_014752952	Bradi1g48340	(Huang *et al.*, 2011)
	BdSWN	XP_010228709	Bradi1g64460	
Cicer arientinum (chickpea)	CaCLF	XP_00451191	-	This study
	CaEZL1	XP_004495901	-	
	CaEZL2	XP_004494933	-	
	CaEZL3	XP_004515047	-	
Glycine max (soybean)	GmCLF1	XP_006573641	Glyma.01G188000	This study
	GmCLF2	XP_006590652	Glyma.11G054100	
	GmEZL1A	XP_003519745	Glyma.02G012100	
	GmEZL1B	XP_006588564	Glyma.10G012600	
	GmEZL2A	KRH68336	Glyma.03G224300	
	GmEZL2B	KRH28654	Glyma.11G067000	
	GmEZL3A	KRG96530	Glyma.19G216600	
	GmEZL3B	KRH68267	Glyma.03G219800	
Medicago truncatulata	MtCLF	XP_003611696	Medtr5g016870	This study
	MtEZL1	XP_003591396	Medtr1g086980	
	MtEZL2	XP_003625973	Medtr7g109560	
	MtEZL3	XP_003622865	Medtr7g055660	
Oryza sativa (rice)	OsCLF	XP_015644234	LOC_Os06g16390	(Luo *et al.*, 2009)
	OsSWN (SET1)	XP_015630972	LOC_Os03g19480	
Pisum sativum (pea)	PsCLF	-	PsCam056516	This study
	PsEZL1	-	PsCam049340	
	PsEZL2	-	PsCam028561	
	PsEZL3	-	PsCam029108	
Phaseolus vulgaris (common bean)	PvCLF	XP_007156782	Phvul.002G017200	This study
	PvEZL1	XP_007145145	Phvul.007G213900	
	PvEZL2	XP_007156927	Phvul.002G029000	
	PvEZL3	XP_007163181	Phvul.001G213300	
Solanum lycopersicum (tomato)	SlCLF1 (EZ2)	NP_001234760	Solyc01g079390	(How Kit *et al.*, 2010)
	SlCLF2 (EZ3)	NP_001234765	Solyc03g044380	
	SlSWN (EZ1)	XP_010315972	Solyc02g093190 +	
Zea mays (corn)	ZmCLF (Mez1)	AAM13420	GRMZM2G157820	(Springer *et al.*, 2002)
	ZmSWN1 (Mez2)	AAM13421	GRMZM5G875502	
	ZmSWN2 (Mez3)	AF443598	GRMZM2G043484	

4.2.4 Sequence details for alignments and phylogenetic analysis of FtsH

Identification of FtsH family members were identified in BLASTp using the predicted protein sequences for known Arabidopsis FtsH genes (Table 4.3). *Medicago* coding sequences identified were then used to tBLASTn to the PsCameor database to retrieve homologous sequences.

Table 4.3 FtsH family cDNA sequences used in alignments and phylogenetic analysis.

Species	Gene Name	Accession	Locus Number	Reference(s)
Arabidopsis thaliana	FtsH1	NM_103909	AT1G50250	Garcia-Lorenzo et. al (2006)
	FtsH2	NM_147357	AT2G30950	
	FtsH3	NM_128465	AT2G29080	
	FtsH4	NM_128172	AT2G26140	
	FtsH5	NM_123592	AT5G42270	
	FtsH6	NM_121529	AT5G15250	
	FtsH7	NM_114573	AT3G47060	
	FtsH8	NM_100523	AT1G06430	
	FtsH9	NM_125277	AT5G58870	
	FtsH10	NM_100625	AT1G07510	
	FtsH11	NM_124696	AT5G53170	
	FtsH12	NM_106604	AT1G79560	
Medicago truncatulata	FtsH1	XM_013606798	Medtr3g115110	This study
	FtsH2A	XM_013607897	Medtr2g438140	
	FtsH2B	XM_013601908	Medtr4g094662	
	FtsH3A	XM_003619527	Medtr6g059690	
	FtsH3B	XM_003623990	Medtr7g078570	
	FtsH3C	XM_013594368	Medtr7g096060	
	FtsH3D	XM_003606639	Medtr4g064350	
	FtsH4A	XM_003603109	Medtr3g104490	
	FtsH4B	XM_013590371	Medtr8g469460	
	FtsH4C	XM_003615941	Medtr5g074850	
	FtsH4D	XM_003615980	Medtr5g075340	
	FtsH4E	XM_013598721	Medtr5g075360	
	FtsH4F	XM_003603108	Medtr3g104480	
	FtsH4G	XM_003603107	Medtr3g104470	
	FtsH6	XM_003621186	Medtr7g010800	
	FtsH7A	XM_003615536	Medtr5g069780	
	FtsH7B	XM_013604712	Medtr3g462930	
	FtsH11	XM_013610911	Medtr1g028390	
	FtsH12	XM_00362835	Medtr8g056860	
	FtsH13A	XM_003602543	Medtr3g096000	
	FtsH13B	XM_013591765	Medtr8g099605	
Pisum sativum	FtsH1	-	PsCam049149	This study
	FtsH2A	-	PsCam025440	
	FtsH2B	-	PsCam044093	
	FtsH3A	-	PsCam036540	
	FtsH3B	-	PsCam033630	
	FtsH4A	-	PsCam037400	
	FtsH4B	-	PsCam020811	
	FtsH4C	-	PsCam056377	
	FtsH4D	-	PsCam013931	
	FtsH4E	-	PsCam020890	
	FtsH4F	-	PsCam012896	
	FtsH4G	-	PsCam033773	
	FtsH6	-	?	
	FtsH7A	-	PsCam014684	
	FtsH7B	-	PsCam049299	
	FtsH11	-	PsCam033718	
	FtsH12	-	PsCam044993	
	FtsH13A	-	PsCam045048	
	FtsH13B	-	PsCam045012	

4.3 Results

4.3.1 Aero1 mapping

Mapping results described in Chapter 3 located *AERO1* between markers *FENR1* and *UNK1* at the bottom of pea LG I, which corresponds to the top of Medicago chromosome 5. This region of the Medicago genome was scanned to identify potential candidate genes, considering homologs of Arabidopsis genes known to have a role in phase change or with more general roles in development and transcriptional regulation. Several potential candidate genes were identified, including *HISTONE ACETLYTRANSFERASE OF THE CBP FAMILY 1* (*HAC1*), *APETALA 2* (*AP2*) and *CURLY LEAF* (*CLF*) (Table 4.4). Both *HAC1* and *CLF* function in epigenetic regulation and their loss-of-function mutants exhibit pleiotropic developmental defects including defects in the timing of flowering (Deng *et al.*, 2007; Goodrich *et al.*, 1997). AP2 belongs to a family of transcription factors that regulates flower development in Arabidopsis and is known to be targeted by multiple pathways, including miR172 genes in the phase change pathway (Jofuku *et al.*, 1994; Zhu & Helliwell, 2010; Jung *et al.*, 2014).

Table 4.4 *AERO1* candidate genes between *FENR1* and *UNK1* flanking markers

Gene Name	Corresponding Medicago Locus
FENR1	Medtr5g022300
HAC1	Medtr5g017020
CLF	Medtr5g016870
AP2	Medtr5g016810
UNK1	Medtr5g016100

The most likely candidate for *AERO1*, among those surveyed, appeared to be *CLF* due its mutant defects described in Arabidopsis being most similar to our pea mutant with pleiotropic effects on leaf morphology, hence the name “curly leaf”, reduced plant height and earlier flowering (Goodrich *et al.*, 1997). Mapping of *CLF* confirmed its location within the interval and revealed its position very close to *AERO1*, with no recombinations identified between *CLF* and *AERO1*, indicating a distance of less than 1cM between *AERO1* and *CLF* (Figure 4.1).

Even though there are no recombinations, the small distance between them on the map is due to a single missing data point in the CLF marker scoring.

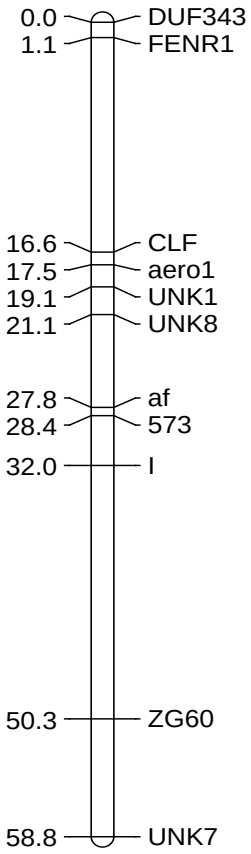


Figure 4.1 Pea linkage group I, showing *CLF* mapping very close to the *AERO1* locus.
The linkage map was constructed from estimations of genetic distance between molecular and morphological markers based on segregation data using JoinMap®4 (Van Ooijen, J.W., 2006; Kyazma B.V., Wageningen, Netherlands). *CLF* marker specifics can be found with the rest of the marker information in the previous chapter (Table 3.2). Population of *TER* x *aero1* (n=60)

4.3.2 CLF gene conservation in pea

CLF cDNA was amplified and sequenced from NGB5839 using primer pairs outlined in the materials and methods section of this chapter (Table 4.1). The sequences were compared with the Medicago and Arabidopsis *CLF* orthologs and confirmed to be highly similar and likely correct. CLF homologs in other species have a number of domains including the highly conserved SET (**S**u(var) **E**(z) **T**rithorax) domain that is required for its methyltransferase activity (Müller *et al.*, 2002; Trievel *et al.*, 2002). Alignments of CLF proteins from Arabidopsis, maize and rice previously revealed several other unique domains in addition to the SET domain including two domains of unknown function (EZD1 and EZD2), a SANT (**S**WI3, **A**DA2, **N**-CoR and **T**FIIIB) DNA-binding domain, and a cysteine-rich region (CXC) (Ng *et al.*, 2007). All of these domains were identified in the pea CLF sequence, revealing conservation of the CLF gene structure in pea consistent with it functioning in epigenetic regulation similar to CLF proteins in other species (Figure 4.2).

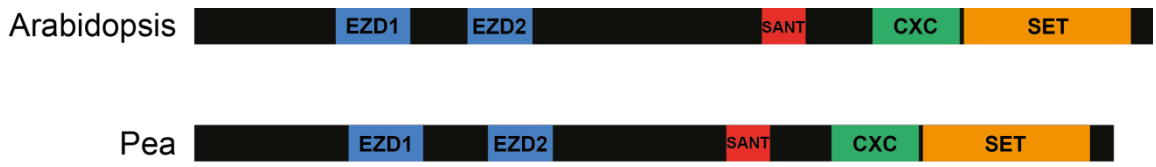


Figure 4.2 Domain conservation between CLF sequences in pea and Arabidopsis
Illustration of the full amino acid sequence for CLF in pea and Arabidopsis (*Arabidopsis thaliana*). The conserved domains are shaded in blue (EZD1 & EZD2), red (SANT), green (CXC) and orange (SET).

4.3.3 Aero1 carries a mutation in CLF

Sequencing of *CLF* cDNA from the mutant *aero1-1* allowed comparison with the WT sequence, which revealed a G1889T transition in *aero1-1* resulting in a cysteine to tyrosine substitution at residue 623, located in the conserved CXC domain (Figure 4.3). The alignment in Figure 4.3 shows that the C623 residue is highly conserved across CLF orthologs from both dicots and monocots, as well as in the CLF homologs from Arabidopsis, SWINGER (SWN) and MEDEA (MEA) (Figure 4.3). A second *aero1* mutant allele, *aero1-10* (Marx, 1986; Taylor & Murfet, 2003), was also found to carry a single nucleotide deletion (T2555Δ) at the 3' end of the coding region downstream from the SET domain. This mutation is predicted to result in a frameshift at codon 852, and termination of translation following the addition of 23 missense amino acids (Figure 4.3).

These two independent deleterious mutations in the *CLF* gene for *aero1-1* and *aero1-10* provide strong evidence that *AERO1* is *CLF* in pea. The respective locations of the mutations also seems to be consistent with the severity of their phenotypes. *Aero1-1* contains a mutation in a conserved domain and has a more severe phenotype than *aero1-10*, containing a mutation outside the conserved domains and positioned towards the end of the protein sequence.

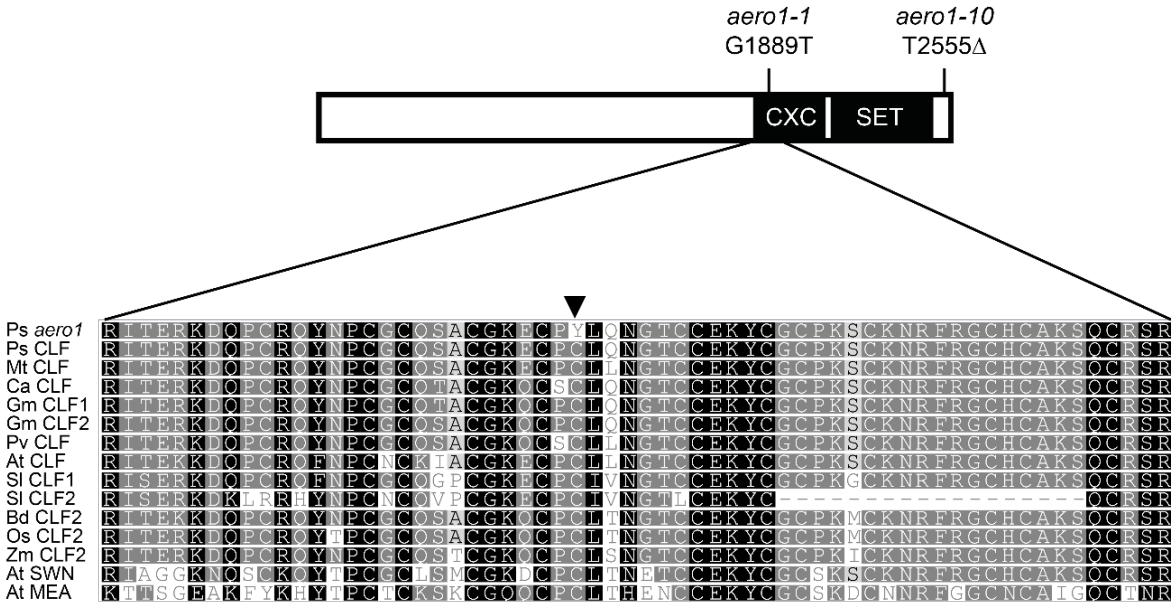


Figure 4.3 SNP in *CLF* for *aero1-1* and *aero1-10*
Diagrammatic representation of the location of the SNPs in the *CLF* gene for *aero1-1* and *aero1-10*. The start of the CXC domain has been enlarged with full protein alignment. The black arrow represents the location of the highly conserved cysteine that has been changed to a tyrosine in *aero1-1*. CLF protein sequences were included from the following species to show conservation of the cysteine highlighted: *Arabidopsis thaliana* (At), *Brachypodium distachyon* (Bd), *Cicer arietinum* (Ca), *Glycine max* (Gm), *Medicago truncatulata* (Mt), *Oryza sativa* (Os) *Phaseolus vulgaris* (Pv), *Pisum sativum* (Ps), *Solanum lycopersicum* (Sl) and *Zea mays* (Zm).

4.3.4 The E(z) protein family in legumes

In view of the likely identity of *AERO1* as the pea *CLF* ortholog, it was of interest to examine the composition and function of the CLF family in pea and other legumes. The *Arabidopsis* CLF protein functions as a component of the Polycomb Repressive Complex 2 (PRC2), which plays a major role in epigenetic regulation of gene expression and acts to modify chromatin through the methylation of histones, effecting transcriptional repression (Goodrich *et al.*, 1997; Schubert *et al.*, 2006). PRC2 belongs to a wider group of Polycomb group complexes (PcG) that were first discovered in *Drosophila* as regulators of homeotic genes, and include PRC1, PRC2 and Pcl-PRC2 complexes (Muller & Verrijzer, 2009). PRC2 is now considered the most structurally conserved of these PcG complexes in plants (Pien & Grossniklaus, 2007; Hennig & Derkacheva, 2009).

PcG proteins were first discovered in plants in 1997, and now it appears that all multicellular plants are likely to have a functional PcG system with multiple complexes that contain overlapping and unique components (Chen *et al.*, 2009; Mosquna *et al.*, 2009). For example, Arabidopsis contains 12 homologs of Drosophila PRC2 subunits (Hennig & Derkacheva, 2009): the three E(z) homologs CLF, MEDEA (MEA) and SWINGER (SWN) ; the three Suppressor of zeste (Su(z)12) homologs EMF2, FERTILISATION INDEPENDENT SEED2 (FIS2) and VERNALIZATION2 (VRN2); the single Extra sex combs (Esc) homolog FERTILIZATION INDEPENDENT ENDOSPERM (FIE); and the five p55 homologs MSI1–5 (Pien & Grossniklaus, 2007; Köhler & Villar, 2008). Evidence suggests that these proteins form at least three PRC2-like complexes: the EMBRYONIC FLOWER (EMF), VERNALIZATION (VRN) and FERTILISATION INDEPENDENT SEED (FIS) complexes (Bemer & Grossniklaus, 2012). FIE functions in all three complexes, CLF and SWN function in both the EMF and VRN complexes, and the rest of the components are unique to a particular complex.

To gain a broader understanding of the CLF/E(z) family in legumes, members of the family were identified in pea and other legumes, and subjected to phylogenetic analysis together with homologs from Arabidopsis, tomato and monocot species (Figure 4.4). These results show that diploid legumes (*Pisum sativum, Medicago truncatula, Cicer arietinum, Phaseolus vulgaris*) have a basic complement of four E(z)-like proteins and the paleotetraploid soybean (*Glycine max*) has the expected eight (Alves-Carvalho *et al.*, 2015; Goodstein *et al.*, 2012). This analysis

shown in Figure 4.4 identified a well-supported clade of CLF orthologs in which all dicot and monocot proteins were very similar and clustered closely. Each of the diploid legumes, including pea, have only a single *CLF* ortholog. Three other pea E(z)-like proteins were also identified and grouped within a well-supported clade of legume E(z)-like sequences which is sister to a clade comprised of the other Arabidopsis E(z) paralogs, MEA and SWN and a single tomato ortholog. Figure 4.4 highlights the fact that although AtMEA is most closely related to AtSWN, it is highly divergent. MEA orthologs appear to be present only in the Brassicaceae lineage (Spillane *et al.*, 2007) and are thought to have undergone rapid evolution and neo-functionalisation.

The legume E(z)-like genes are therefore, strictly speaking, not orthologs of either SWN or MEA, and it seems most appropriate to refer to them simply as E(z)-like (EZL). The legume EZL clade contains two distinct subclades, EZL1 and 2. The EZL1 proteins are highly conserved, whereas the EZL2 proteins are much less conserved and *EZL2* genes appear to have undergone independent duplications within both the warm season phaseoloid and the temperate galegoid legumes (Figure 4.4). This suggests that the EZL1 proteins may be more similar to CLF in their function, and may also participate in the PRC2 complex, whereas the EZL2 and EZL3 group proteins may have more divergent functions. Given these various duplication events, it is also possible that, there may be some degree of redundancy among the legume EZL genes, or as in the case of Arabidopsis MEDEA, neo-functionalisation may have also occurred.

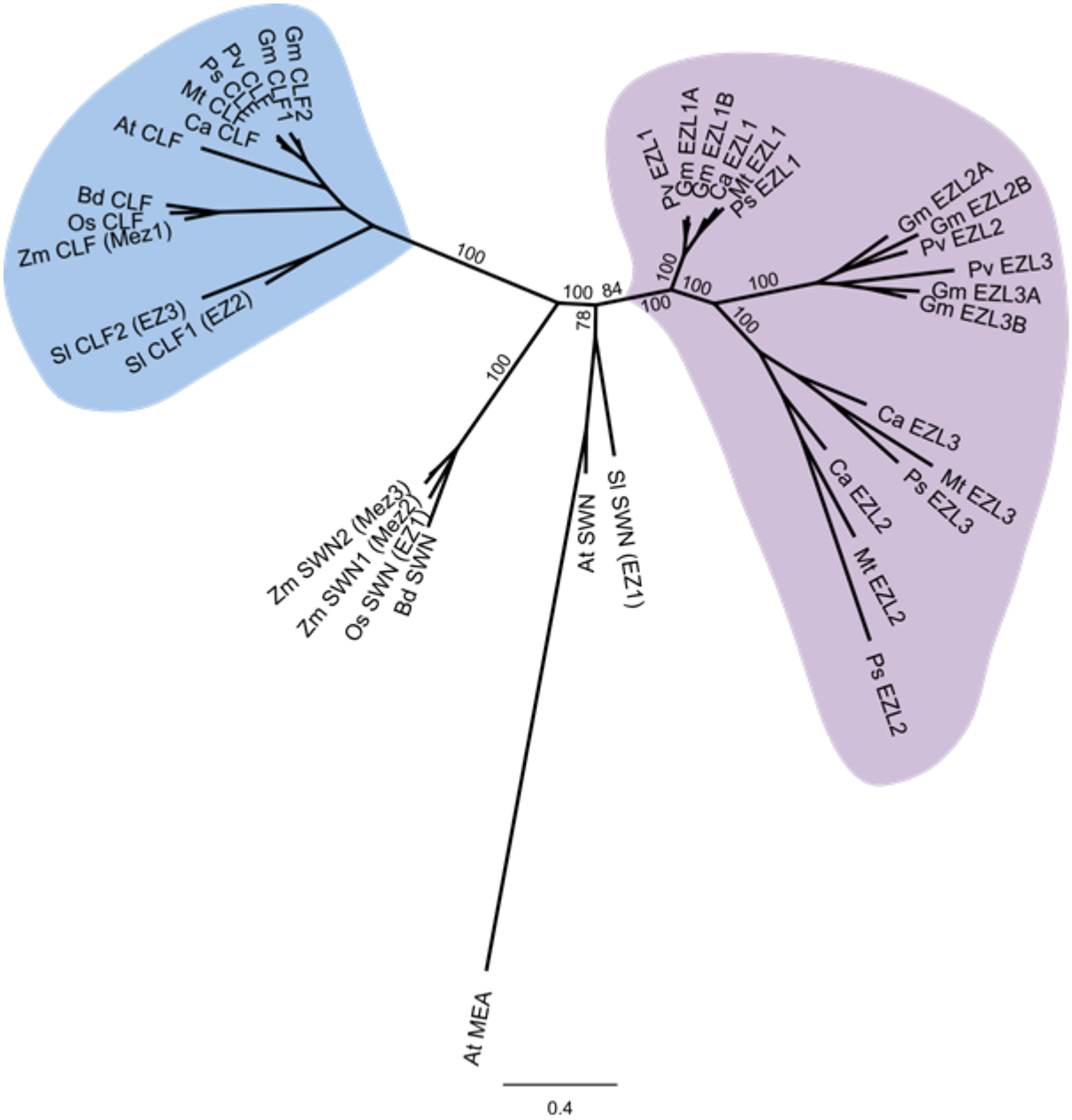


Figure 4.4 Phylogenetic maximum likelihood tree of E(z) related proteins

The unrooted radial phylogram was constructed from full length predicted protein sequence for all E(z) family genes in *Arabidopsis thaliana* (At), *Brachypodium distachyon* (Bd), *Cicer arietinum* (Ca), *Glycine max* (Gm), *Medicago truncatulata* (Mt), *Oryza sativa* (Os) *Phaseolus vulgaris* (Pv), *Pisum sativum* (Ps), *Solanum lycopersicum* (Sl) and *Zea mays* (Zm). The CLF clade is shaded blue and the legume specific EZL clade in purple. Bootstrap values obtained from 100 trees are indicated next to critical branches. Sequence information can be found in Table 4.2.

4.3.5 APC1 mapping

Chapter 3 described a significant refinement of the map position for *APC1*, narrowing its location to a 10 cM interval between markers *RPL28* and *TAF* in LGII, which corresponds to a region at the top of Medicago chromosome 1. Attempts to further refine the map position of *apc1* using the Medicago comparative mapping approach, encountered several inconsistencies. Markers that were expected to map in the region of interest, actually mapped at a distance, much closer to the top of pea LGII. Previous mapping studies in pea had reported the inversion of a section of pea LGII relative to Medicago chromosome 1 (Aubert *et al.*, 2006; Bordat *et al.*, 2011) and this result was confirmed in our population. As further markers were added, it also emerged that *APC1* was situated right at the junction of this inversion and some of the markers targeting pea orthologs of Medicago genes in the region of this junction did not map on pea LGII at all. To provide additional insight into the possible gene order in pea, the corresponding region was also identified in other legume genomes. Chickpea in particular looked useful since comparative mapping results among other legume species and pea showed that chickpea chromosome 4 was co-linear with pea LGII in the same orientation (Tayeh *et al.*, 2015)Figure 4.5).

In chickpea the interval between *RPL28* and *TAF* orthologs contained approximately 600 genes, but since the *APC1* locus mapped much closer to *RPL28* than to *TAF*, only the 200 genes closest to *RPL28* were systematically screened. This analysis identified two potential candidate genes; *Nuclear pore complex protein 85* (*NUP85*), and *Fertilisation Independent Endosperm* (*FIE*) (Table 4.5). Nuclear pore proteins are vital for plant growth and development as they transport molecules between the cytoplasm and the nucleus, and mutants of some *NUP* genes display multiple defects (Meier & Brkljacic, 2009). In legumes, NUP85 has so far been found to have a role to play in nodulation and seed production (Saito *et al.*, 2007). *FIE*, like *CLF*, is another polycomb complex gene involved in plant developmental processes, including seed development, repression of flowering during the vegetative phase and vernalisation. (Katz *et al.*, 2004).

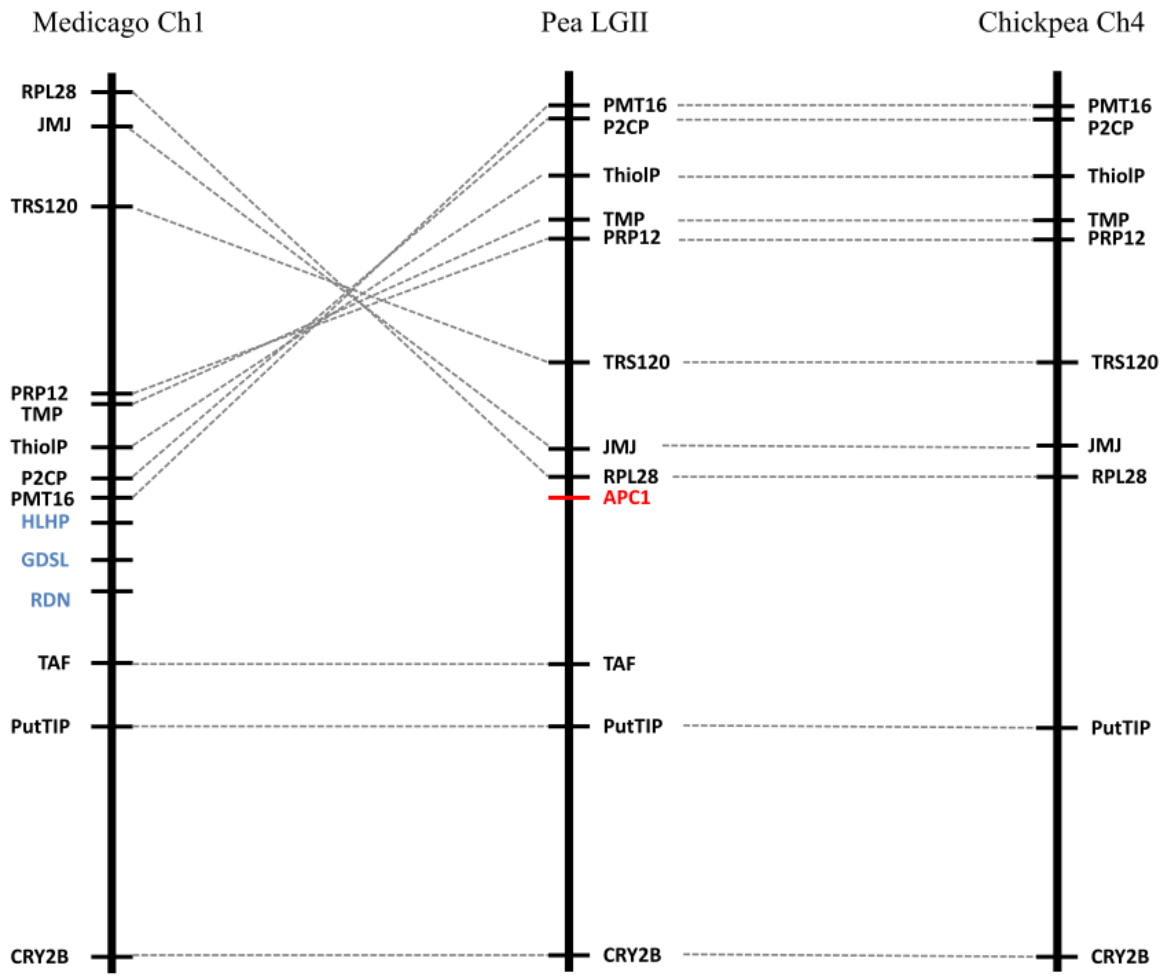


Figure 4.5 Comparative mapping data for Medicago, pea and chickpea.
A diagrammatic representation of the loci positions mapped in this study in pea and their corresponding locations in Medicago (*Medicago truncatulata*) and chickpea (*Cicer arietinum*). Markers designed from Medicago, but did not map on LGII in pea are coloured in blue. The APC1 locus, investigated in this study, is coloured in red. TAF in chickpea is currently located on an unmapped scaffold.

Table 4.5 APC1 candidate genes between RPL28 and TAF flanking markers.

Gene Name	Corresponding Medicago Locus	Corresponding Chickpea Gene ID
RPL28	Medtr1g007480	101511437
NUP85	Medtr1g006690	101503070
FIE	Medtr1g028310	101507081
TAF	Medtr1g033390	101497596

Transcripts for pea orthologs were identified from the reference transcriptome and both genes were mapped relative to *APC1*. In a population of 261 plants, the *NUP85* marker segregated closely with the *apc1* phenotype, but a single recombination ruled it out as a candidate. To confirm this result, the full-length cDNA was sequenced in WT and *apc1* and was found to be identical (Table 4.1). In the same population, the *FIE* marker showed perfect co-segregation with *apc1*, consistent with the possibility that *FIE* could be the causal gene for *APC1*. However, sequencing of the full length *FIE* cDNA also revealed no polymorphism between WT and *apc1* (Table 4.1). This result suggests that *FIE* is not the correct candidate, but also that *APC1* must be very close to the *FIE* location.

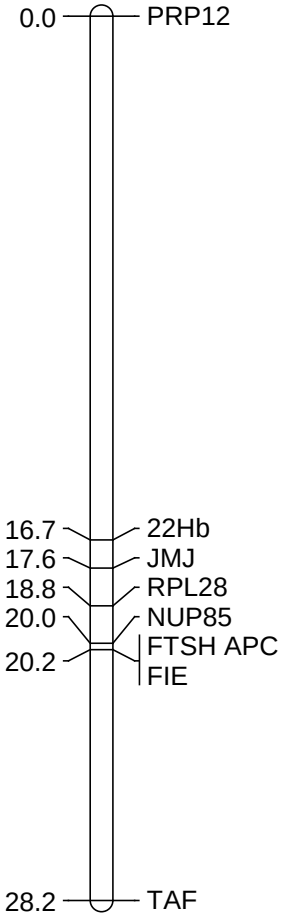


Figure 4.6 Pea linkage group II, showing APC1 mapping with FIE and FTSH.
The linkage map was constructed from estimations of genetic distance between molecular and morphological markers based on segregation data using JoinMap®4 (Van Ooijen, J.W., 2006; Kyazma B.V., Wageningen, Netherlands). APC1 marker specifics can be found with the rest of the marker information in the previous chapter (Table 3.4). Population of TER x *apc1* (n=261).

4.3.6 Analysis of RNAseq data in apc1 *reveals a mutation in* FtsH11

By this stage of the project, the opportunity to use an RNAseq approach to search for SNPs in *apc1* became available. As part of a larger project to explore the utility of RNAseq for identification of SNPs between WT and mutants, RNAseq data were generated from several mutants (including *apc1*) by Dr V. Hecht and were provided for analysis. For analysis of *apc1*, a reference was constructed consisting of approximately 200 full-length pea cDNAs identified as the probable orthologs of Medicago genes in the region immediately below NUP85 (Medtr1g006690) in BLAST searches of the pea gene atlas (Alves-Carvalho *et al.*, 2015). RNA-seq (Wang *et al.*, 2009) reads were mapped to this reference and individual transcripts were systematically examined for the presence of SNPs. Unfortunately, this preliminary experiment provided only relatively low depth, and many of the genes in the region did not have either full coverage or enough depth to compare mutant and WT transcript sequences effectively. Nevertheless, careful analysis of the available data revealed a polymorphism between WT and *apc1* in transcript PsCam033718, encoding a protein belonging to the filamentation temperature-sensitive metalloprotease (*FtsH*) family (Yu *et al.*, 2004). In chickpea (Varshney *et al.*, 2013), this gene is located within six genes of *FIE*. The SNP was located in exon 8 and consisted of a G1211A transition predicted to result in the substitution of an arginine with a histidine at residue 404. This was determined from 18 WT reads (all G) and 3 *apc1* reads (all A). The presence of the SNP was confirmed by Sanger sequencing from WT and *apc1* and the inferred location of the *FtsH* gene was confirmed by mapping in the TER x *apc1* population, which revealed perfect co-segregation of *FtsH* and *APC1* (Figure 4.6). As designing a marker around the putative causal SNP proved difficult, mapping of the *FtsH* candidate was done using a different SNP identified between NGB5839 and TER.

Genetic analysis of the *apc2* mutant in Chapter 3 indicated that this mutant, although very similar in phenotype to *apc1*, was likely to represent a distinct locus, as markers near *APC1* had no association with leaf change phenotype in an *apc2* mapping population. Nevertheless, to provide further evidence against the possibility that *apc1* and *apc2* might be allelic, the *FtsH* gene was sequenced from the *apc2* mutant. Primers were designed to sequence the full 17-exon cDNA, and RNA was extracted from 3-day-old embryo shoots (Table 4.1). Sequencing revealed no SNPs in *apc2* compared to the wild-type NGB5839.

4.3.7 The FtsH protein family in pea.

The identification of a potential causal SNP for the *apc1* mutant was a promising result, but also difficult to interpret due to the novel nature of the *FtsH* gene and the lack of significant amount of information that could explain its functional relationship to the observed *apc1* phenotypes. The function and phylogenetic relationships within the *FtsH* family were therefore examined.

The FtsH family is a versatile group of proteins in plants that can function as both chaperones and proteases (Akiyama *et al.*, 1994; Leonhard *et al.*, 2000). They contain a single AAA domain, which contains two motifs (the Walker A and B motifs) necessary for nucleotide binding and hydrolysis, and a so called 'second region of homology' (SRH) that carries conserved arginine residues important for oligomerisation and nucleotide hydrolysis (Figure 4.7) (Ogura & Wilkinson, 2001; Ogura *et al.*, 2004; Bieniossek *et al.*, 2009). FtsH proteins also contain a protease domain C-terminal to the AAA domain categorized as a Zn^{2+} metalloprotease domain. The FtsH protein is anchored to the cytoplasmic membrane where it forms hexameric complexes with other FtsH proteins (Moldavski *et al.*, 2012).

In Arabidopsis, the *FtsH* family consists of 12 genes. To gain insight into the FtsH family in legumes and the possible significance of the identified SNP, BLAST searches with Arabidopsis *FtsH* cDNA sequences were used to identify Medicago and pea sequences in the Phytozome and PsCameor databases (Table 2.3; 4.3). These analyses identified 21 FtsH proteins in Medicago and 19 in pea. Alignments confirmed that all FtsH proteins have a conserved structure including the presence of both the AAA and protease domains. One pea sequence was truncated by 50% at the 5' end, suggesting possible incorrect assembly or an otherwise incomplete nature. Since genomic data was unavailable for comparison, this sequence was excluded from the alignment and any phylogenetic analysis.

The SNP discovered between WT and *apc1* in PsCam033718 is located in the conserved AAA domain between the Walker A and Walker B signature regions (Beyer, 1997), which is the narrow central pore region of the FtsH hexamer (Yamada-Inagawa *et al.*, 2003). Interestingly, the arginine residue affected by the SNP is not strongly conserved, and is only present in FtsH11 proteins. However, even in the diverse Arabidopsis FtsH family no other member has a histidine in that position, other mutations studied in the central pore region between the

Walker A and Walker B regions exhibit mutant phenotypes that have reduced ATPase activity (Sakamoto *et al.*, 2004), and previous research has revealed that arginine residues in parts of the AAA domain are vitally important for the ATPase activity of the gene (Karata *et al.*, 1999). Combined, these results suggest it is possible the SNP in *apc1* has impaired the function of FtsH, but it is not likely to be a complete knockout phenotype.

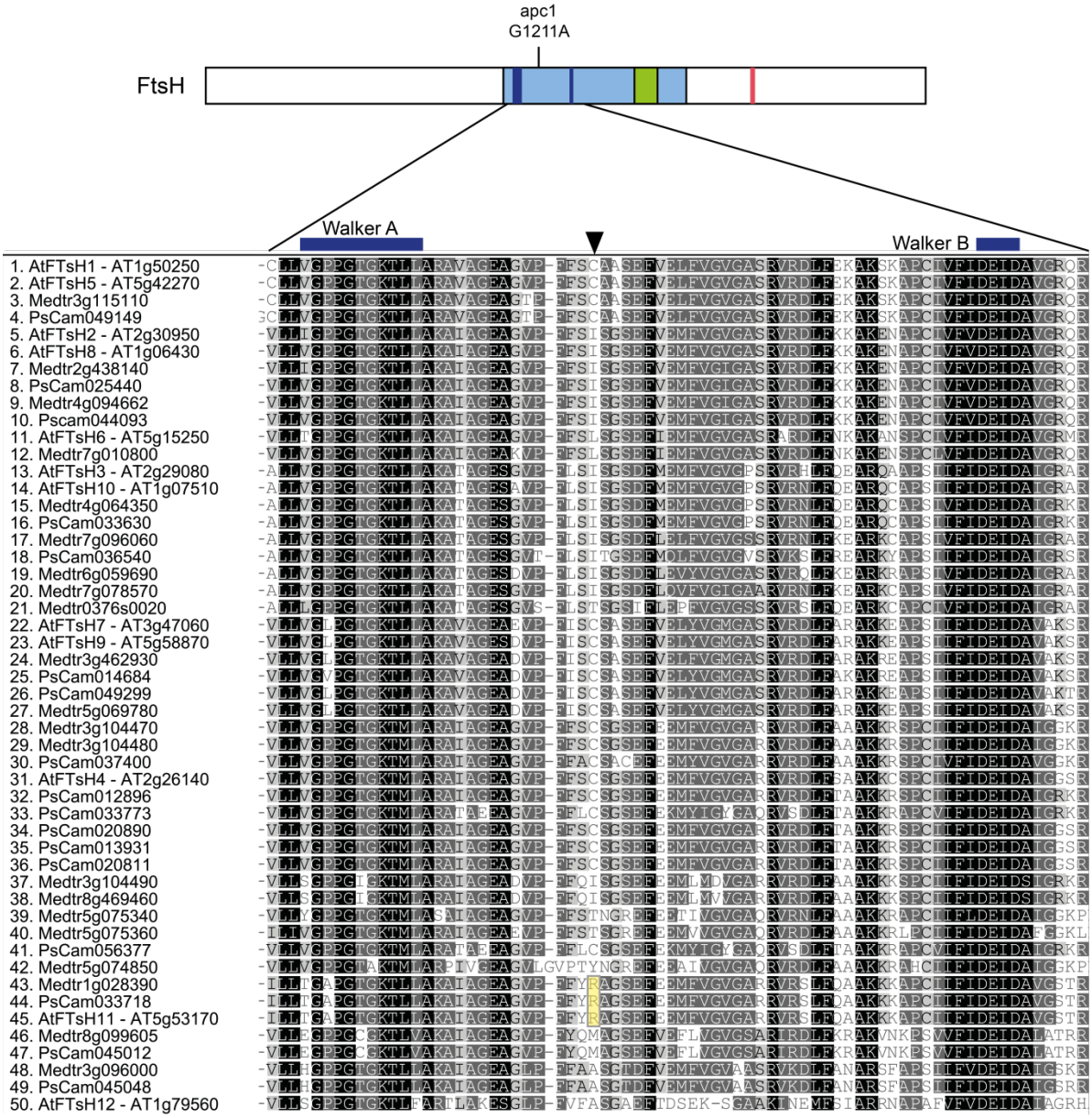


Figure 4.7 Illustration of an *FtsH* gene with its conserved domains and the SNP found in pea *FtsH11* (PsCam033718).

The protein sequence of the start of the AAA domain (light blue) for all pea, Medicago and Arabidopsis FtsH genes is shown. The Walker A and Walker B sequences are highlighted in dark blue. The 'second region of homology' (SRH) towards the end of the AAA domain is highlighted in green and the Zn^2 metalloprotease domain in red. The triangle points to the amino acid that is changed in *apc1* from an Arginine (R) to a Histidine (H) in pea FtsH11 (highlighted in yellow in the sequence).

To further extend understanding of the legume FtsH family, phylogenetic analysis was performed on the entire family in pea, Medicago and Arabidopsis (Table 4.6; Figure 4.8). These results show eight clades that illustrate various patterns of gene duplication or loss in the legumes and unambigiously identified PsCam033718 (the putative *APC1* gene) as the ortholog of Arabidopsis *FtsH11*.

There are three clades (*FtsH6*, *FtsH11* and *FtsH12*) that have single orthologous genes for pea and Medicago, although the pea ortholog was not found for *FtsH6*. However, this gene is most likely represented by the truncated pea sequence mentioned above. Two clades (*FtsH2*/*8* and *FtsH7/9*) appear to have independent duplications in legumes, and in clade 1 (*FtsH1*/*5*), there appears to have been a duplication event in Arabidopsis, but not in legumes. Two further clades show a more extensive expansion of the legume *FtsH* family: four *FtsH3* genes were identified in Medicago, but only two in pea, indicating that a possible tandem duplication event has occurred after divergence of the Trifolieae and Viceae tribes. Confirmation of this would require information from additional legume species in each taxon, such as lentil and clover, and also galegoid species basal to both clades, such as chickpea. Interestingly, there were seven paralogous *FtsH4* genes in both Medicago and pea. In Medicago, at least five of these genes appear to be arranged in two tandem arrays on chr3 and 5, so more detailed analysis and mapping of pea genes will be needed to work out how much of this expansion is common to pea and Medicago and how much has occurred independently in the two species. Sequence analysis suggests these are all unique sequences, but may possibly be pseudogenes, and it will be interesting to observe if this expansion is also present in other legume species. In any case, the duplication events in *FtsH4* suggest that significant redundancy may exist for this gene in legumes.

In view of the multifunctional nature of FtsH genes, and their ability to act as either chaperones or proteases (Akiyama *et al.*, 1994; Leonhard *et al.*, 2000), it is not unexpected that the roles discovered for FtsH genes in Arabidopsis are varied. These include thermotolerance, photosystem repair, organelle development, leaf development, quality control of membrane proteins, and proteolysis (Bailey *et al.*, 2002; Sakamoto *et al.*, 2002, , 2004; Chen *et al.*, 2006; Gibala *et al.*, 2009; Wagner *et al.*, 2016). Of all the FtsH genes in Arabidopsis for which mutants have been described, it is *ftsh4* mutants, rather than *ftsh11*mutants that show aberrations most comparable to pea *apc1*. These include leaf morphology irregularities, an asymmetric rosette shape suggesting phyllotaxy issues, and a developmental delay in the

appearance of true leaves under particular conditions (Gibala *et al.*, 2009; Smakowska *et al.*, 2016). However, the Arabidopsis FtsH4 protein is most closely related to, and is known to form complexes with, FtsH11 (Urantowka *et al.*, 2005). Thus, it is possible that pea APC1 may be functioning in a manner more similar to FtsH4 in Arabidopsis.

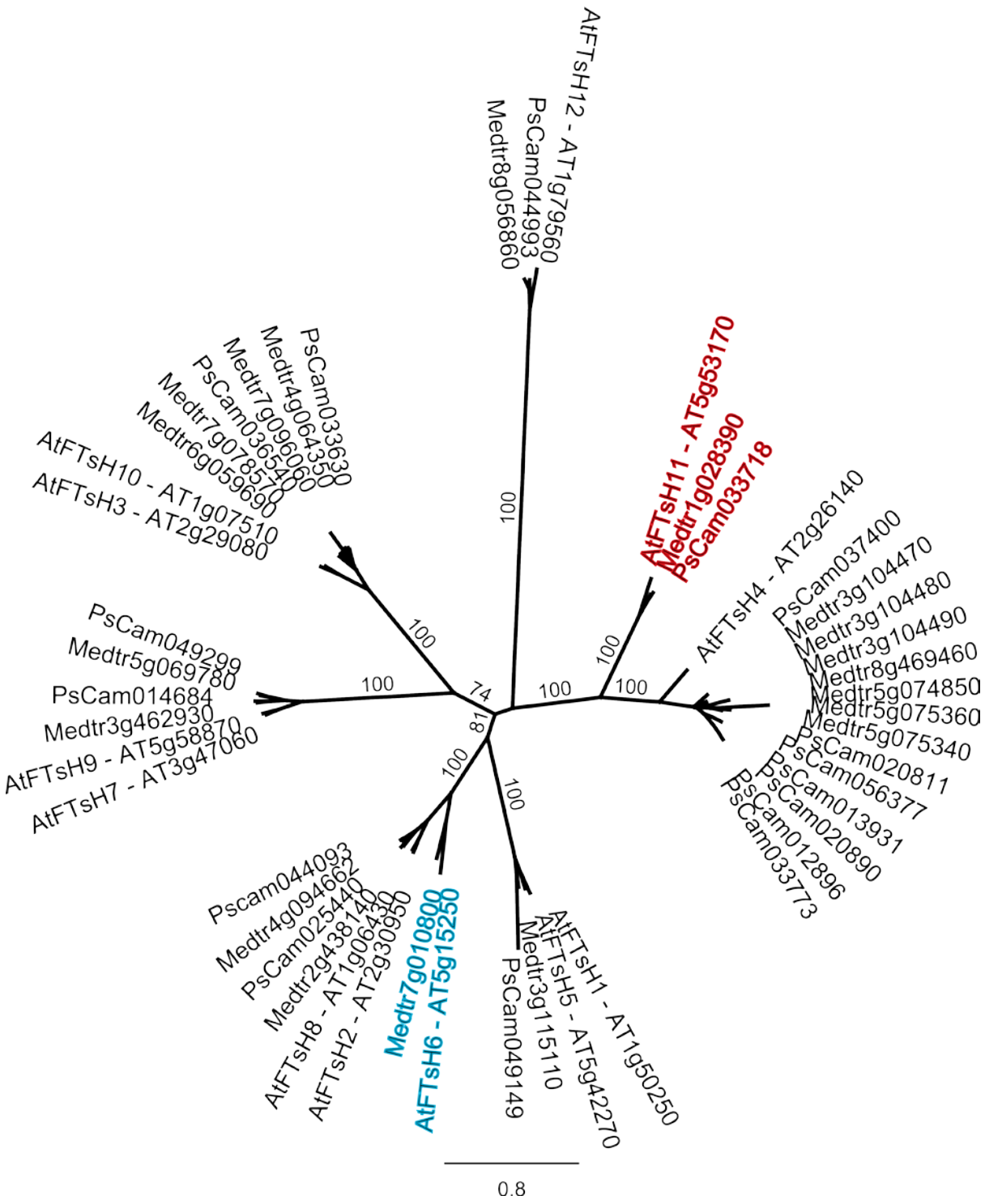


Figure 4.8 Maximum-likelihood tree of FtsH related sequences

The unrooted radial phylogram was constructed from full length cDNA nucleotide sequence for all the active (containing Zn-binding motif) FtsH family genes in *Arabidopsis thaliana* (At), *Medicago truncatulata* (Mt) and *Pisum sativum* (Ps). Bootstrap values obtained from 100 trees are indicated next to critical branches. The pea sequence (PsCam033718) that is mutated in *apc1* is highlighted in the red clade. The clade of the putative missing pea FtsH is highlighted in blue. Sequence information can be found in Table 4.3.

Table 4.6 Phylogenetic clades of *FtsH* genes for Arabidopsis and the number of genes in each clade for Medicago and pea. Clades are named according to the lowest number for Arabidopsis *FtsH* in that clade.

Clade	Arabdiopsis FtsH	Medicago FtsH	Pea FtsH
1	AtFtsH1, 5	1	1
2	AtFtsH2, 8	2	2
3	AtFtsH3, 10	4	2
4	AtFtsH4	7	7
6	AtFtsH6	1	?
7	AtFtsH7, 9	2	2
11	AtFtsH11	1	APC1
12	AtFtsH12	1	1

4.3.8 Expression profiling of pea FtsH genes

Additional information in the PsCam database provides expression profiles for all the cDNA contigs available using the wild-type line cv.Cameor (Alves-Carvalho *et al.*, 2015). The APC1 candidate PsFtsH11 in pea is expressed throughout the plant, with particularly high levels in the young shoot, leaf and apex, suggesting it plays a role in diverse aspects of plant development (Figure 4.7). Other pea genes *FtsH1*, *FtsH2A*, *FtsH2B*, *FtsH7A* and *FtsH7B* have a similar expression pattern to FtsH11 (Table 4.1). Interestingly, when the expression profiles of the numerous pea *FtsH4* paralogs were checked, only *FtsH4D* and *FtsH4F* had medium to high expression levels throughout the plant, with rest showing no or very little expression. This adds weight to the argument that pseudogenisation may have occurred during or after gene duplication events in *FtsH4*, rendering most of these genes non-functional.

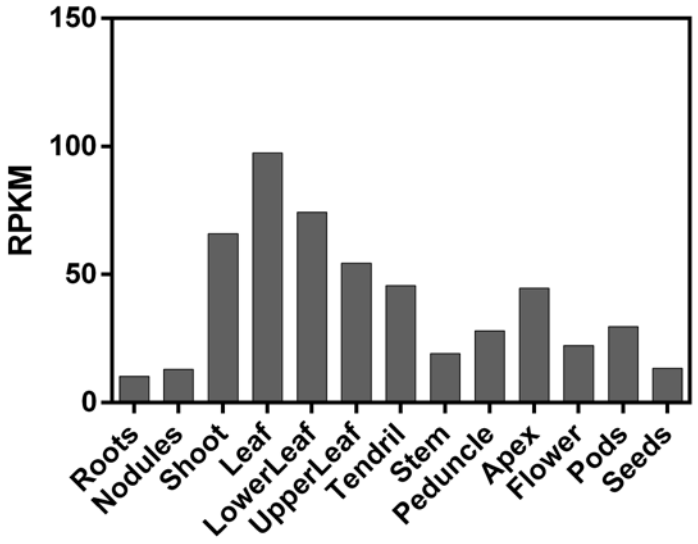


Figure 4.9 Expression profile of pea *FtsH11*/*APC1*
Reads Per Kilobase Million (RPKM) calculated from RNA-seq data in (Alves-Carvalho *et al.*, 2015). This profile shows the expression of *FtsH11*/*APC1* in different plant tissues.

4.4 Discussion

The results presented in this chapter provide important new evidence about the molecular identity of the *AERO1* and *APC1* loci based on comparative mapping and candidate gene analysis. They represent the first breakthroughs in isolating genes for mutants affecting the leaf change trait. One goal in undertaking this work was to ascertain whether these loci could be known components involved in the vegetative phase change pathway. Interestingly, this seems not to be the case for either locus. The results also provide intriguing insights into the genetic control of the leaf change phenotype in pea, but also add further uncertainty about how this may relate to phase change.

4.4.1 AERO1/CLF

Among several candidates within a narrow mapping interval, the *aero1-1* mutant was found to carry a mutation in the *CLF* gene, a well-known plant gene involved in epigenetic regulation. A second allele, *aero1-10*, with a slightly weaker phenotype, also carried a mutation in *PsCLF* gene. The severity of the mutation corresponded well with the severity of the mutant phenotype and the fact that an independent and functionally significant mutation was found to affect *CLF* in each of the *aero1* mutant alleles, strongly supporting the likely identity of *AERO1* as *PsCLF*. To further strengthen the case it should be straight-forward in future to sequence one or more of the other eight known *aero1* alleles.

CLF proteins are a well-known epigenetic regulators of gene expression that are deeply conserved across the plant kingdom. CLF proteins are histone modifying enzymes that act as part of the PRC2 complex to repress target genes by trimethylating histone H3 lysine 27 (H3K27me3) (Schubert *et al.*, 2006). Consistent with this general role in epigenetic regulation, CLF proteins control diverse aspects of plant development. The *clf* mutant in Arabidopsis was named after its curled leaves, a conspicuous feature of its phenotype (Goodrich *et al.*, 1997). CLF also directly represses *FLC*, *FT* and *AG* expression, thereby delaying flowering and floral organogenesis in Arabidopsis (Goodrich *et al.*, 1997; Schubert *et al.*, 2006; Jiang *et al.*, 2008). Despite the loss of repression of *FLC* in the *clf* mutant, which should theoretically delay flowering in the absence of vernalisation, it is likely that the early flowering phenotype of the *clf* mutants is due to the additional de-repression of FT along with other genes that promote flowering such as AGL19 (Schonrock, 2006; Jiang *et al.*, 2008). The *ez2 (clf1)* mutant in tomato has also been characterised (Boureau *et al.*, 2016) and has dramatic effects on

vegetative development, fruit development and ripening but does not affect flowering. While the likely identity of *AERO1* as *PsCLF* was somewhat unexpected, it is perhaps not surprising in view of the various conserved and divergent roles for CLF in different species.

As an epigenetic regulator, CLF function is not confined to one genetic pathway, but involves modifying the expression of many genes across the genome. Chromatin Immunoprecipitation & DNA sequencing (ChIPseq) technology has been used in both Arabidopsis and tomato to identify H3K27me3-associated regions across the entire genome. The orthologous Arabidopsis *clf* and tomato *ez2* mutants both reveal a global reduction in the levels of methylation marks, showing that CLF proteins are vital for this epigenetic function and cannot effectively be complemented by the other related E(z)-like genes in these two species (Boureau *et al.*, 2016; Lafos *et al.*, 2011). Given that the normal mode of CLF action is to cause genome-wide modification of histones by adding suppressive methylation marks to many targets, it is therefore likely that AERO1 also has multiple targets across the genome and affects numerous developmental pathways.

Unfortunately, the identity of *AERO1* as *PsCLF* does not immediately shed much light on the mechanisms of vegetative phase change, or indeed provide any evidence on the question of whether the leaf change phenotype is a manifestation of phase change. However, it does suggest that there is a significant epigenetic component to its regulation. This is not to say that AERO1 does not regulate components of the phase change pathway. In fact, there is growing evidence in Arabidopsis that epigenetic regulation may be involved in control of the transitions between difference phases. At the broad level, multiple modes of epigenetic regulation have been implicated in developmental transitions, including DNA methylation, histone modification and histone-variant deposition (reviewed in Wollmann & Berger, 2015). In all cases the mutants exhibit pleiotropic defects, in line with their broad epigenetic functions. One example is the DNA methyltransferase gene, *MET1*, in Arabidopsis. Mutations lead to a genome-wide reduction in cytosine methylation, resulting in a phenotype that includes late flowering and a heterochronic delay in the juvenile to adult rosette leaf transition (Kankel *et al.*, 2003).

It is also interesting to consider whether there is any precedent for epigenetic regulation of the phase change pathway. Vegetative phase change phenotypes have not been reported for the Arabidopsis *clf* mutant, but genome-wide analyses of H3K27me3 marks showed that 83% of

miR156, 80% of *miR172*, 9% of *SPL* and 33% of *AP2* genes were H3K27me3 targeted (Lafos *et al.*, 2011). These authors went on to investigate the expression of miR156 target genes *SPL3*, *SPL5* and *SPL9* in a *clf*/*swn* double mutant, finding no change in expression of *SPL5* and *SPL9*, and a reduction in *SPL3*. However, if CLF and SWN act to add repressive marks, the expression of their target genes in the double mutant would be expected to increase, not decrease. This is suggested to reflect a parallel reduction in H3K27me3-dependent repression of *miR156* in the double mutant, which would mean *miR156* expression stays high, suppressing SPLs. Thus it is possible that CLF might act to repress multiple targets of the phase change pathway and these effects might cancel each other out in the earlier stages of development, resulting in no net effect.

To answer the question whether the phenotype of accelerated leaf change and flowering time in *aero1* could reflect alteration of the phase change pathway, one approach would be to examine whether expression of these genes is altered in pea *clf* mutants compared to the wild-type either through targeted analyses of *miR156* and *SPL* genes, or through the use of RNA sequencing to look at expression changes across the whole transcriptome. This might also help to identify additional targets of *AERO1*. Another possibility would be to perform a ChIP-seq analysis to identify which genes and genomic regions are subject to CLF-dependent H3K27 trimethylation. Of particular interest would be comparing these results to Arabidopsis and tomato H3K27me3 regions. However, both RNAseq and ChIP methods are costly and technically challenging, particularly in pea, so the feasibility and benefit of such studies would need to be clear before being undertaken. It is also likely that there may be difficulties in interpreting results from these large scale experiments given that CLF may be targeting many different genes and developmental pathways at the same time.

Characterisation of the E(z) family in legumes, to which CLF belongs, has provided greater understanding of the history of these genes in legume evolution. In Arabidopsis, the three E(z) related proteins, AtCLF, AtSWN and AtMEA are very closely related, making study of their functions particularly challenging (Hennig & Derkacheva, 2009). In particular, the functional redundancy between SWN and CLF has impaired the analysis of their specific functions. The phenotype of the *clf* single mutant has curly leaves, floral defects and earlier flowering, but the *swn* single mutant has not visible phenotype. However, the severe defects of the *clf swn* double mutants reveal the partial functional redundancy between these two genes (Chanvivattana, 2004; Schubert *et al.*, 2005). With respect to MEA, the situation observed in pea and Medicago

mirrors all other plants analysed so far, with MEA being unique to the Brassicaceae lineage and an outlier in phylogenetic analysis (Spillane *et al.*, 2007).

This analysis revealed that AERO1/CLF is a single-copy homolog of Arabidopsis CLF, but there do not seem to be any direct homologs in pea and Medicago for AtSWN. It is likely that legumes have evolved their own unique clade of E(z)-like genes, whose roles are yet unknown. Multiple duplication events for the E(z) family in pea and Medicago also increase the complexity in legumes. Since CLF and SWN have partially redundant functions in Arabidopsis, it is likely that this may also be the case in pea. Due to the deeply conserved nature of the EZL1 clade in legumes, it is possible that pea EZL1 might play a similar role to CLF and putatively function in the same or similar PcG complex. Further redundancy may also exist if the other EZL proteins in pea also have similar functions to CLF, or alternatively, they may have entirely divergent functions, as seen in the recent duplication in the Brassicaceae lineage for AtMEA. Thus, pea may provide another unique system in which to study the roles of E(z) proteins in plants. Finding mutants in these other E(z) related genes, including creating double and triple mutants, would be of great interest for future research.

4.4.2 APC1/FtsH11

Results in this chapter also identified the Filamentation temperature-sensitive metalloprotease gene *FtsH11* as a possible candidate for *APC1*. Evidence for this comes from the lack of recombination in a large mapping population and the presence of a SNP directing an amino-acid substitution in the *apc1* mutant. However, given the fact that the map interval still contains approximately 500 genes, the RNAseq did not provide complete coverage of these genes, and that no other *apc1* mutant alleles are available, the identity of *APC1* as *FtsH11* remains provisional. The most direct way to strengthen this evidence would be to obtain additional mutant alleles of *FtsH11* by reverse genetics using the pea TILLING platform (http://www-urgv.versailles.inra.fr/tilling/pea.htm; (Dalmais *et al.*, 2008). Additional support could come through the isolation of mutants for Medicago *FtsH11* from the *Tnt1* insertion platform (https://medicago-mutant.noble.org/mutant/; (Tadege *et al.*, 2008).

In view of the prominent and relatively specific leaf change phenotype of the *apc1* mutant, our initial hypothesis was that that *APC1* might have a fairly central role in controlling vegetative

phase change pathway, and would most likely be a known component of this pathway. The possible identity of *APC1* as *FtsH11* is therefore unexpected. As yet, there is no known role for FtsH genes in any aspect of phase change in any plant species. Assuming it can be proven that *APC1* is *FtsH11*, one possible interpretation is that *FtsH11* is a target or upstream regulator of components of the phase change pathway, such as *SPL* genes. Alternatively (as discussed above for *AERO1*) the phenotype of leaf change is not indicative of phase change at all, but of some other underlying process affecting leaf development in which *FtsH11* has a role. Undoubtedly, future results will reveal that simple conclusions do not tell the full story and the reality is far more complex.

The FtsH proteins are a family of membrane-bound proteases containing an AAA-ATPase domain associated with various cellular activities, and a Zn^{2+} metalloprotease domain with a proteolytic function (Beyer, 1997; Arnold & Langer, 2002). Multiple FtsH proteins come together to form complexes, that are homo-hexameric or hetero-hexameric in plants (Akiyama *et al.*, 1995; Moldavski *et al.*, 2012). Arabidopsis contains 12 genes coding for active FtsH proteases, which operate in the mitochondria (Janska *et al.*, 2010), in the chloroplasts (Ferro *et al.*, 2010), or in the case of FtsH11, possibly in both (Urantowka *et al.*, 2005). The role of FtsH proteins in plant development are diverse, given that they operate both as chaperone molecules, transporting molecules through organelle membranes, and in regulated protein degradation (reviewed in (Janska *et al.*, 2010; Wagner *et al.*, 2012).

Several of the Arabidopsis FtsH genes have been functionally characterised. The *VAR1*/*VAR2* group is named after the variegated leaf phenotype of loss-of-function mutants *ftsh1*, *2*, *5* and *8*. These mutants are highly sentsitive to photosystem II photo damage and show a leaf-variegation phenotype with two sectors in the same leaf: green sectors containing normal chloroplasts and white sectors where chloroplast development is arrested (Bailey et al., 2002; Sakamoto et al., 2002, 2004; Kato et al., 2009). The *ftsh11* mutant has a chlorotic phenotype when grown in long days, suggesting problems with chloroplast structure and function as well. It also exhibits reduced photosynthetic capacity and arrested development when exposed to high temperatures, giving evidence that FtsH11 is crucial for thermotolerance (Chen *et al.*, 2006; Wagner *et al.*, 2016). Interestingly, the FtsH11 protein is most closely related to, and is known to form complexes with FtsH4 (Urantowka *et al.*, 2005) and an *ftsh4* mutant shows morphological defects that are more similar to those of *apc1* in pea. These include irregular leaf serrations (which could represent a defect in growth potential of the leaf), an asymmetric

rosette shape suggesting phyllotaxy defects, and a developmental delay in the appearance of true leaves under higher temperatures in long days and under normal temperatures in short days (Gibala *et al.*, 2009; Smakowska *et al.*, 2016). These phenotypes suggest that pea APC1 may be functioning in a more similar role to FtsH4 in Arabidopsis, except that the timing of leaf development is accelerated in *apc1*, not delayed.

Very little has been uncovered for *FtsH* genes in other species. A single pea FtsH was described by (Kolodziejczak *et al.*, 2002), to be closely related to FtsH3/10 in Arabidopsis and on closer inspection of the sequence, it matches PsCam033630. Through immunoblot analysis it was found to operate exclusively in the mitochondria and had the ability to complement the respiration deficiency of yeast *yta10* and *yta12* mutants, suggesting it is a functional homologue of the yeast mitochondrial protease. Incorrectly, (Janska *et al.*, 2010) then went onto suggest pea has only one mitochondrial matrix associated FtsH, which our phylogenetic results contradict. Analysis of the Medicago *FtsH1* gene expression revealed that exposing plants to low temperatures or to high light conditions increased FtsH mRNA and protein levels in the leaves (Ivashuta *et al.*, 2002). In widely divergent roles, the tobacco homolog of AtFtsH5 was shown to be involved in defence against pathogens (Seo *et al.*, 2000) and in capsicum, the corresponding gene was found to be involved in chromoplast vesicle fusion and possibly membrane biogenesis (Hugueney *et al.*, 1995). Therefore, it may not be surprising that FtsH11 in pea has other divergent functions also. Among the known roles and functions of FtsH proteins in Arabidopsis and other plants, there is little to suggest how a mutation in an FtsH gene might cause an acceleration of the timing of leaf change in pea. If the pea mutant *apc1* is indeed defective in FtsH11 function then the effects on leaf development might represent a novel role for this gene in pea compared with other species.

Given that FtsH4 and FtsH11 are part of a small group of FtsH proteins that operate in the mitochondria, it could be possible that the defects in organ development seen in both *apc1* and *Atftsh4* are related to energy production by the mitochondria, and possibly chloroplasts since FtsH11 operates in both. During plant growth, the cell division and expansion required to produce leaves relies on energy produced by organelles. In Arabidopsis, mutants with impaired FtsH4 expression have mitochondria that produce less ATP due to an increase in oxidative stress (Smakowska *et al.*, 2016). The mutation for *apc1* also resides in the AAA domain, required for its ATPase activity, adding further weight to this argument that energy production may be affected in these mutants. However, an increase in compound leaf complexity (and

therefore leaf biomass) in the *apc1* mutant would seem to signify an increase in energy production, not a decrease, and as such would not be consistent with the reduction in energy production expected if *APC1*, like *AtFtsh4* had a role in generating ATP (Smakowska *et al.*, 2016). These speculations will need to be investigated in future research to determine the molecular nature of the mutation in *apc1* causing the leaf change phenotype.

Finally, phylogenetic results and expression profile analyses also gave additional information about the FtsH family of proteases in legumes. Since FtsH proteins are known to form hexamer complexes with other FtsH proteins (Moldavski *et al.*, 2012), it is possible that these other pea FtsH proteins could function with APC1 in a complex. It will also be worth investigating whether *apc2*, with a very similar mutant phenotype to *apc1*, may be defective in one of these other similarly expressed *FtsH* genes. It also opens up possible new candidate genes to consider in attempts to identify the other locus *AERO2* characterised in chapter 3.

Chapter 5

Genomic characterisation of the miRNA156-SPL module in pea

5.1 Introduction

MicroRNA156 (miR156) and its target *SPL* genes are highly conserved in the plant kingdom, and together they form a gene regulatory network that controls various aspects of plant growth and development (Huijser & Schmid, 2011; Wang & Wang, 2015). The transition from the juvenile to the adult phase of shoot development in plants is accompanied by changes in vegetative morphology and an increase in reproductive potential. Recent studies suggest that miR156 is necessary and sufficient for the expression of the juvenile phase, and regulates the timing of the juvenile-to-adult transition by coordinating the expression of functionally distinct *SPL* transcription factors, that control different aspects of this process (Wu *et al.*, 2009). The characterisation of *aero* and *apc* mutants presented in Chapters 3 and 4 has left open the question of whether these mutants, or the phenomenon of leaf change itself, have any relationship to the miR156/SPL module. This chapter aims to begin the characterisation of this module in pea by the characterisation of these gene families.

5.1.1 The microRNA156 family

MiRNAs represent one of several types of endogenous non-coding small RNAs, but only represent a very small fraction of the total number of small RNAs in plants (Lu *et al.*, 2005). They are 20-22 nucleotides in length and regulate gene expression at the post-transcriptional level by mRNA cleavage or translation inhibition (Bartel, 2004; Schwab *et al.*, 2005). They are encoded mainly in intergenic regions of the plant genome and, unlike animal miRNAs, employ near-perfect complementary sequences to affect their targets (Rhoades *et al.*, 2002; Schwab *et al.*, 2005; Nozawa *et al.*, 2012).

The process of generating functionally mature miRNAs is complex, involving multiple steps and enzymes, and it is this process that allows them to be distinguished from other small RNAs (Voinnet, 2009). MiRNA transcripts, generated by DNA-dependent RNA polymerase II (Pol II) (Lee *et al.*, 2004; Parizotto, 2004), are derived from longer, self-complementary precursor

RNAs (pre-RNA) that form pseudo-double-stranded hairpin structures (Meyers *et al.*, 2008). A duplex of approximately 21 nucleotides is excised from the hairpin precursor by a DICER-LIKE (DCL) protein and subsidiary proteins (Park *et al.*, 2002; Kurihara & Watanabe, 2004). Each strand of the duplex is protected via methylation by HUA ENHANCER 1 (HEN1) (Park *et al.*, 2002) before it is exported from the nucleus and then one strand of the duplex is excised and incorporated into a complex containing an ARGONAUTE (AGO) protein, becoming a mature miRNA (Hutvagner & Simard, 2008; Vaucheret, 2004). The other strand of the duplex is generally excluded from association with AGO and degraded (Bologna *et al.*, 2012; Rogers & Chen, 2013). MiRNAs have diverse precursor sequences, even within specific families, but the mature miRNA remains highly conserved in sequence and size.

MiR156 is one of the most ancient miRNAs and is conserved across flowering plant species (Axtell & Bowman, 2008; Morea *et al.*, 2016). Phylogenetic analyses suggest that the most of the individual miR156 loci evolved through duplication events, potentially resulting in functional redundancy (Maher, 2006). However, research has revealed that, although the mature miRNA can be identical, the members of a miR156 family have very different spatiotemporal expression patterns, with expression depending on the developmental stage, types of tissue and growing conditions of the plant (Axtell & Bartel, 2005; Xie, 2006). This gene family has been studied in a wide range of crop species including maize (Mica, 2006), tomato (Yin *et al.*, 2008; Zhang *et al.*, 2011), rice (Jiao *et al.*, 2010; Xie, 2006), potato (Bhogale *et al.*, 2014) and soybean (Zhang *et al.*, 2008) and a range of non-crop species including Arabidopsis (Xie, 2005), English ivy, blue gum (Poethig, 2013) and Canadian poplar (Wang *et al.*, 2011). Historically, the 20-nucleotide miR156 was distinguished from the 21-nucleotide miR157 (Reinhart, 2002), but due to their almost identical target potential they are now referred to as one family.

There are currently 10 known members of the miR156 family in Arabidopsis and their precursor sequences vary in size from approximately 70-200bp in length. They exclusively target the *SPL* gene family with perfect, or near perfect, sequence complementarity (Rhoades *et al.*, 2002; Wu & Poethig, 2006; Gandikota *et al.*, 2007; Wu *et al.*, 2009). Across all species studied to date, miR156 expression levels are high in the early stages of plant development and decline with age. Vegetative phase change is initiated by the decline in expression of miR156 and the consequent increase in the expression of *SPL* genes. (Wu *et al.*, 2009). When miR156 expression is blocked in Arabidopsis, the resultant plants exhibit a precocious adult phenotype

(Wang, Czech, *et al.*, 2009; Wu *et al.*, 2009). Conversely, when miR156 is overexpressed, the juvenile phase is prolonged and flowering is delayed (Wu & Poethig, 2006).

5.1.2 Squamosa promoter binding-like (SPL) *genes*

SPL genes have been identified in nearly all terrestrial plants, from single-celled green algae and moss to gymnosperms and angiosperms (Cardon *et al.*, 1999; Lännenpää *et al.*, 2004; Arazi *et al.*, 2005; Kropat *et al.*, 2005; Salinas *et al.*, 2012; Zhang *et al.*, 2016). They function in important regulatory roles in plant growth and development, including leaf development, phase change, flower and fruit development, GA signalling and response to copper and fungal toxins (Cardon *et al.*, 1997; Stone *et al.*, 2005; Wu & Poethig, 2006; Zhang *et al.*, 2007; Schwarz *et al.*, 2008; Usami *et al.*, 2009; Yu *et al.*, 2012; Padmanabhan *et al.*, 2013), and also include several agronomically important traits in crops like branching, grain filling and tillering (Jiao *et al.*, 2010; Wang *et al.*, 2012; Aung *et al.*, 2014, , 2015; Zhang *et al.*, 2017). As previously mentioned, *SPL* transcription factors, regulated by miR156, are key influencers of the transitions between developmental phases (Schwab *et al.*, 2005; Wu & Poethig, 2006).

All *SPL* genes feature the highly conserved Squamosa promoter-binding (SBP) domain, which is approximately 79 amino acids long and contains a novel zinc finger motif with two Zn^{2+} binding sites: Cys-Cys-His-Cys and Cys-Cys-Cys-His (Yamasaki *et al.*, 2004). The SBP domain is essential for binding the cis-regulatory element TNCGTACAA (Cardon *et al.*, 1997, 1999), with GTAC as its critical core (Birkenbihl *et al.*, 2005). A putative nuclear localization signal (NLS) is located at the C-terminal of the SBP domain, which partly overlaps with the DNA-binding domain, particularly with the second Zn^{2+} binding structure (Birkenbihl *et al.*, 2005). In Arabidopsis, there are 17 SPL proteins, and as in other species, they vary greatly in size, with some being as short as 131 amino acids in length (AtSPL3), and others as long as 927 amino acids (AtSPL12) (Cardon *et al.*, 1999). Although SPL genes have different numbers of exons, the SBP domain of all land plants is encoded across the first and second exons (Guo *et al.*, 2008).

SPL genes also vary with respect to the presence of a target site for miR156 where miR156 binds to the *SPL*, suppressing its expression (Schwab *et al.*, 2005; Wu & Poethig, 2006; Gandikota *et al.*, 2007; Wu *et al.*, 2009). In *SPLs*, these complementary miR156 sites lie downstream of the SBP domain at the 3' region of the gene, either within the last exon or

within the 3' untranslated region (UTR) (Gandikota *et al.*, 2007), but are present in only a subset of *SPL* genes. For example only 11 of the 17 Arabidopsis SPLs and 11 of the 19 rice SPLs contain miR156 binding sites (Rhoades *et al.*, 2002; Xie, 2006). For all the studied SPLs in Arabidopsis, the GUGCUCUCUCUCUUCUGCA polynucleotide is conserved in the miR156 binding site within the *SPL* mRNA, with the exception of *SPL3*, *SPL4* and *SPL5* that contain three, one and two mismatches respectively at the 3' end of the miRNA (Gandikota *et al.*, 2007). Interestingly, these particular *SPLs* all have their binding sites uniquely located in the 3'UTR. Although the miRNA binding site position relative to the reading frame should not affect its function, all are conserved in the same reading frame, namely the ALSLLS hexapeptide of the SPL protein.

The regulation and function of *SPL* genes has been extensively studied in Arabidopsis. Initial investigations characterised overexpression phenotypes of *SPLs* using either miR156-resistant versions or the constitutive Cauliflower Mosaic Virus 35S promoter. The phenotypes produced suggested that *SPL2-5*, *9-11*, *13* and *15* all controlled phase change, along with various other aspects of shoot development (Cardon *et al.*, 1997; Wu & Poethig, 2006; Wang *et al.*, 2008; Usami *et al.*, 2009; Wang, Czech, *et al.*, 2009; Wu *et al.*, 2009; Yamaguchi *et al.*, 2009; Yu *et al.*, 2010; Wei *et al.*, 2012; Stief *et al.*, 2014). However, in some of these cases, the loss-of-function mutants were unavailable or did not show any obvious phenotype, making it difficult to know whether the over-expression phenoytpes reflected normal functioning of the genes. One example was the overexpression of *SPL3*, which showed an acceleration of abaxial trichome production, suggesting an acceleration of vegetative phase change, and an early flowering phenotype, but the loss of function mutants in the same *SPL3* gene had no obvious phenotype (Cardon *et al.*, 1997; Wu & Poethig, 2006).

The recent, comprehensive study of (Xu *et al.*, 2016) compared expression patterns and phenotypes of miR156-resistant and sensitive *SPLs* and also determined the phenotypes of loss-of-function mutants in individual *SPLs* and in combination. In summary, *SPL2*, *9-11*, *13* and *15* appear to contribute to both vegetative and reproductive phase change, with *SPL9*, *13* and *15* being particularly important. In contrast to previous expression results suggesting that *SPL3*, *4* and *5* regulate phase change (Cardon *et al.*, 1997; Wu & Poethig, 2006; Gandikota *et al.*, 2007), it is now clear that they do not play a major role in either vegetative or reproductive phase change, but do promote the floral meristem identity transition (Xu *et al.*, 2016; Yamaguchi *et al.*, 2009).

It is of interest to compare these results in Arabidopsis to other species to understand whether the functions of orthologous *SPL* genes may be conserved. Silencing of the *AtSPL3/4/5* ortholog in snapdragon, *AmSPB1*, did not affect the timing of flowering, but did interfere with flower fertility, which led to abnormal vegetative phenotypes due to loss of apical dominance and an increase in lateral branching (Preston & Hileman, 2010). In addition, recent silencing of two orthologs of *AtSPL3/4/5* from petunia (*PhSBP1* and *PhSBP2*) also revealed a role for these genes in promoting inflorescence development and flower emergence, similar to snapdragon and Arabidopsis, but also a novel effect on leaf initiation rate (Preston *et al.*, 2016). Further, a reduction of expression of the tomato *AtSPL3/4/5* homolog *LeSPL-CNR*, as a result of an epimutation in its promoter, resulted in impaired fruit ripening (Manning *et al.*, 2006), revealing a novel and divergent function for this SPL in tomato also. In concert, these results reveal that *SPL* orthologs can have similar, but also divergent functions and understanding the role of these in other plant species will be of significant interest.

5.1.3 The miR156-SPL module in legumes

There have been some initial, limited studies to identify and partially characterise miR156 genes in several legume species, including Medicago, Lotus and soybean (Dezulian *et al.*, 2005; Zhang *et al.*, 2008; Jagadeeswaran *et al.*, 2009; Song *et al.*, 2011; Wang *et al.*, 2015; Tripathi *et al.*, 2017). The expression pattern reported for a miR156 sequence in soybean is similar to those in other species, being high in the juvenile stage and declining with age (Yoshikawa *et al.*, 2013). However, this study only looked at a single miR156 and it is unclear whether precursor or mature miRNA sequences were used for expression analysis. The phylogenetic relationships of this sequence to other legume miRNA sequences was not specified.

Functional studies of miR156 in legumes have been limited to overexpression analysis, which reveal varying effects in different species. Transgenic *Lotus japonicus* plants with ectopic expression of *LjmiR156a* showed enhanced branching, delayed flowering, underdeveloped roots and reduced nodulation (Wang *et al.*, 2015). Overexpression of *MsmiR156d* in alfalfa (*Medicago sativa*), also resulted in enhanced shoot branching, and a delay in flowering. However, unlike in Lotus, the alfalfa plants had increased root length, but nodulation remained unchanged (Aung *et al.*, 2014). Finally transgenic soybean plants overexpressing *GmmiR156b* showed a strong delay in flowering time in long days (i.e. non-inductive conditions) only, but

effects on other traits were not discussed (Cao *et al.*, 2015). These results show that particular miR156 in different legumes do play a vital role in plant development and phase change, but they are difficult to compare because the phylogenetic relationships of these different miR156 genes in legume species has not been clearly established.

Relatively little is known about *SPL* genes in legumes, although recent studies, including an evolutionary overview of *SPLs*, have provided partial descriptions of the *SPL* family in *Medicago* and soybean (Aung *et al.*, 2014; Gao *et al.*, 2016; Preston & Hileman, 2013). A recent comprehensive study in soybean identified 41 *SPL* genes of which 17 were found to have miR156 complementary sites (Tripathi *et al.*, 2017). A previous study showed downregulation of the soybean orthologs of *AtSPL3* and *SPL9* in *35S:MIR156b* soybean plants, but other *SPLs* were not studied (Cao *et al.*, 2015). In transgenic alfalfa expression of *SPL2*, *SPL3*, *SPL4 SPL6*, *SPL9*, *SPL12* and *SPL13* was reduced in miR156 overexpression lines, but results varied greatly due to the transgenic lines having diverse transcript abundance of anywhere between eight-fold to 650-fold compared to the control line (Aung *et al.*, 2014).

5.1.4 Chapter Aims

Despite the apparently conserved nature of the miRNA156-SPL module in plants and its importance for vegetative phase change, relatively little work has been done in legumes and none at all in pea. The aim of the work presented in this chapter was to identify and isolate the miRNA156 and *SPL* gene families in pea, to perform phylogenetic analyses of these genes together with those from other legumes and Arabidopsis, and to carry out an initial expression analysis to determine where they may be expressed and how their expression may change during development. This information will be important for further understanding of these gene families, particularly in legumes.

5.2 Materials and Methods

This section contains specific details of materials and methods for research included in this chapter. General materials and methods that are also relevant are described in Chapter 2. Details of online resources are given in chapter 2 (Table 2.3).

5.2.1 qRT-PCR analysis

The results of qRT-PCR analysis that are presented in Figure 5.3 and Figure 5.7, investigating gene expression across a developmental series, were generated from the same expression experiment. Apex and leaf tissue samples were harvested from wild-type (NGB5839) plants at seven day intervals at the same time of day until 5 weeks after germination. Plants were grown in controlled-environment growth cabinets under 20°C and LD photoperiod (24h, fluorescent light). Each apex sample comprised the main stem apices from two plants, dissected to approximately 2-3mm to remove excess stem and developing leaf tissue. Each leaf tissue sample comprised leaflets from the uppermost fully expanded leaf from two plants. Two replicates were collected for each genotype at each time-point. Tissue harvest and the processing of one replicate of leaf and apex samples were completed prior to commencement of this study by Valerie Hecht. During this study, the second replicate of was processed and gene expression was investigated for miR156 precursor transcripts on one replicate and SPL transcripts on both replicates. Primer details are available below in Section 5.2.3.

5.2.2 Primer Details

Table 5.1 Primer used for qPCR of miR156 precursor genes

Gene	Primer Names	Primer Sequences	Tm (°C)	Source
ACT	PsACT-F PsACT-R	GTGTCTGGATTGGAGGATCAATC GGCCACGCTCATCATATTCA	59	(Foo *et al.*, 2005)
miR156A	PsMIR156A-1F PsMIR156A-1R	GCACTGCACCTTACGGAGAG CCATGTTGTGTCGGAGTTTGATG	60	This study
miR156C	PsMIR156C-1F PsMIR156C-1R	TTAAGGGTAAGGGCGGTGAC AGAGAGATGGTGGTGGGGTG	60	
miR156E	PsMIR156E-1F PsMIR156E-1R	TGTTTAAGAGGGAGAGGGAGGA GAGCACAAAGGAAATGAAATGCA	60	
miR156H	PsMIR156H-1F PsMIR156H-1R	TGGCAAGAAGGGTTGAATTGG GGCAAAGGTGGAAAGTCTAGTG	60	
miR156K	PsMIR156K-1F PsMIR156K-1R	TTGCCATGAGAGGTTGAGGC TGCATGAGAAGGGTGATGGTG	60	

Table 5.2 Primers used for qPCR of SPL genes

Gene	Primer Names	Primer Sequences	Tm (°C)	Source
ACT	PsACT-F PsACT-R	GTGTCTGGATTGGAGGATCAATC GGCCACGCTCATCATATTCA	59	(Foo *et al.*, 2005)
SPL2A	SPL2A-1F SPL2A-1R	CATCATCTCTTTCTTCATCGCCC CTCGGAAAAGCAAATGGGCC	60	This study
SPL2B	SPL2B-1F SPL2B-1R	ATGGAAGTGGTTGCTCTGGG ACAGAAGAAAGCTCCGGTGC	60	
SPL3A	SPL3A-1F SPL3A-1R	TGAGGGAAAGAGAAGCTATGAGT GCTTACCACCACTTAGATCAGC	60	
SPL3B	SPL3B-1F SPL3B-1R	TTTGAAGAGGAGGAGGAAGG ATCTCTGCAATGTGTACGGC	61	
SPL3C	SPL3C-1F SPL3C-1R	CTGTTGTGATGGTTGCAGGG AGGTTGATGTTGTCCTTTGCC	61	
SPL6A	SPL6A-1F SPL6A-1R	CGCACTTCCACTTTACCTTCC CGCTTATCATCATCGAACTCAGC	61	
SPL6B	SPL6B-1F SPL6B-1R	GTGAATTTGATGATGGTAAGCGC AGATGCTTGTGGTGGCTTGG	61	
SPL6C	SPL6C-1F SPL6C-1R	ATTCACTCAGGAAGGGCTGG CTAGATCCGGTTCCGTCTTCG	60	
SPL9A	SPL9A-1F SPL9A-1R	GAGCTCCGTGTTTACCTCGC TTGTTTGATCGCCGCGTACC	60	
SPL9B	SPL9B-1F SPL9B-1R	CCAACTCTCTCTTAACTTCGCG CGGGTGTTGTGGTTTGATTTCC	60	
SPL13A	SPL13A-1F SPL13A-1R	ACTTCATTCCAAGACTCCTGAGG TCAGGCTGAGGTTTTCTTCTCC	60	
SPL13B	SPL13B-1F SPL13B-1R	GCAGGAAACGTTTAGATGGGC ACATCAGTTCCACCCCAAGTAG	60	
SPL13C	SPL13C-1F SPL13C-1R	TCCTGTTGTGTTGGTTGGAGG AGAGAAGGTGGTTGAGGTTTCC	60	
SPL20	SPL20-1F SPL20-1R	TAGGAGAAGACTTGCAGGGC GCACTGCACCTTACGGAGAG	60	

5.3 Results

5.3.1 Identification of the miRNA156 *gene family in Medicago and pea*

To identify pea miR156 sequences, the Medicago *miR156* precursor sequences were obtained from miRBase ((Kozomara & Griffiths-Jones, 2014); http://www.mirbase.org/cgi-bin/query.pl?terms=mtr-miR156&submit=Search) and tBLASTn against GenBank pea TSA sequence and nucleotide databases. BLAST searches of the pea transcriptome were also employed (Alves-Carvalho *et al.*, 2015), but very few sequences were recovered, most likely due to stringent parameters used for the original sequence analysis that effectively excluded most small RNAs from the dataset. However, some relevant sequences were identified from GenBank. Table 5.3 shows all the Medicago mature miR156 sequences (a-k), their duplex sequences and the genomic locations of their precursor sequences. Partial analyses of the Medicago family have been reported in previous papers (Dezulian *et al.*, 2005; Jagadeeswaran *et al.*, 2009; Szittya *et al.*, 2008), but the aim in this study was to re-examine and update these results to provide a comprehensive overview for this particular miRNA family as a solid basis for comparison with pea sequences. The precursor sequences in Medicago are all located in intergenic regions, except for *miR156a*, which seems to originate from the opposing strand of the genomic section of an *SPL* gene (*SPL20*).

Alignments of the pea precursor sequences obtained with the Medicago precursor sequences (Table 5.3), was achieved using CLUSTALW (Larkin *et al.*, 2007). From these results, the mature miRNA156 and their duplex sequence positions were identified from the homologous Medicago sequences. They were also checked for the ability to form a stem loop with the mature and duplex sequences aligning, using the online stem loop prediction tool RNAfold (Figure 5.2; http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi; (Lorenz *et al.*, 2011). The pea precursor sequences all formed stem loops with the usual features; a stem section containing the mature miRNA and its complementary sequence (the duplex) and a looped section and ends that are subject to Dicer-catalysed excision (Figure 5.2; (Bartel, 2004).

A possible new miR156 sequence was also uncovered in BLAST searches for pea miR156. The mature miR156 and duplex sequences had a small number of nucleotide differences and did not match any of the other pea sequences (Table 5.3). When the precursor sequence was used in turn to query the Medicago database, it was found not to match any of the previously

identified *miR156a-j* sequences and to have a novel location on chromosome 3. This new putative miR156 precursor gene was designated as *miR156k* (the next available letter in the sequence) for both pea and Medicago (Figure 5.1; Table 5.3). It has an identical mature sequence to *miR156a* (Figure 5.1), however the stem loop and duplex sequences are very different.

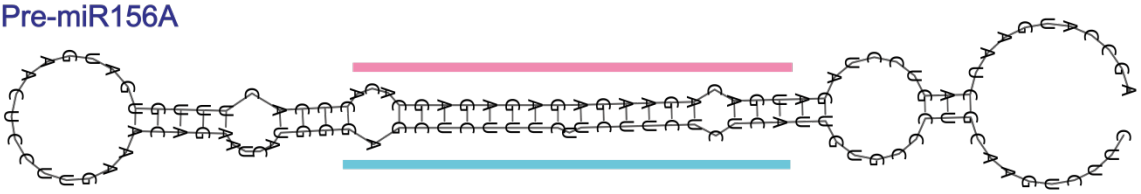
Figure 5.1 The pea mature miRNA156 sequences

Table 5.3 The Medicago miRNA156 family and the pea matches

Name	mature miRNA	duplex	Ch	Exact genome location of precursor	Locus location between	Pea Sequence
miR156a	TGACAGAAGAGAGAGAGCACA	GAGCTCTTTCTTCTTCTCTCA	7	15012922-15013381	7g444860 (SPL20)	PsCam037459 (SPL20)
miR156b	TGACAGAAGAGAG**T**GAGCAC	GTGCTCACTCTCTATCTGTCA	1	4043023-4043114	1g015780 & 1g015810	GAMJ01065257 / JI935192
miR156c	TGACAGAAGAGAG**T**GAGCAC	GTGCTTACTCTCTATCTGTCA	3	48230226 - 48230323	3g104640 & 3g104660	JI897432
miR156d	TGACAGAAGAGAG**T**GAGCAC	GTGCTCACTCATCTTTCTGTCA	3	48955732 - 48955821	3g106080 & 3g106100	-
miR156i	TGACAGAAGAGAG**T**GAGCAC	GTGCTCACTTCTCTTTCTGTCA	4	54672426 - 54672518	4g131063 & 4g131080	-
miR156j	TGACAGAAGAG**G**G**T**GAGCAC	GTGCTCATACTCTTCTGACA	1	3310108 - 3310210	1g013670 & 1g013660	-
miR156e	**T**TGACAGAAGA**T**AGAGAGCAC	GTGCTCTCTATGCTTCTGTCAT	8	44922531 - 44922702	8g106450 & 8g106470	JI901233
miR156f	**T**TGACAGAAGA**T**AGAGAGCAC	GTGCTCTCTGCTCTTCTGCCAA	6	7611795 - 7611888	6g021970 & 6g022000	-
miR156g	**T**TGACAGAAGA**T**AGAG**G**GCAC	GTGCTCTCTAGACTTCTGTCA	6	30617131 - 30617250	6g082000 & 6g082020	-
miR156h	**T**TGACAGAAGA**T**AGAGAGCAC	GTGCTCTTTATTCTTCTGTCA	7	31748920 - 31749013	7g082780 & 7g082800	JI908611 / JI897483
miR156k	TGACAGAAGAGAGAGAGCACA	GTGCTCTCCCTTCTTCTGTCAT	3	23939448 - 23939578	3g460810 & 3g460830	PsTT0041391

AGCCATGAATCAGTCCTAGATGACAGAAGAGAGAGAGCACACCCACTTTGTGATGAACTCCTTGA
AACAGAATCATGGGAGCTCTTTCTTCTTCTCTCATTGTGCCCTGCAAGTCTTC

Pre-miR156A



GGAGGTGACAGAAGAGAGTGAGCACACATTGGTATTTTCTTATTATGATATTTCATATTTGAAGCT
ATGCGTGCTCACTTTCTATCTGTCACCCCGT

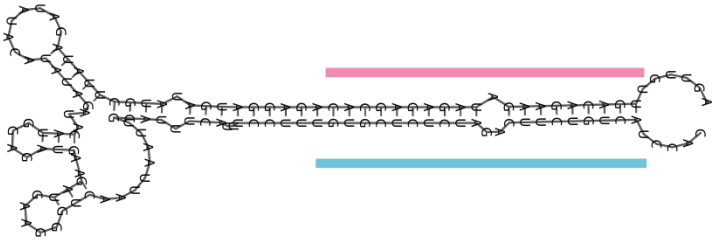
Pre-miR156B

GGCGGTGACAGAAGAGAGTGAGCACACATGGTGGTTTTCTTGCATGATGATGGTTCATGCTTGA
AGCTATGCGTGCTCACCCTCTATCTGTCACCCCAC

Pre-miR156C

AGTTGTTGACAGAAGATAGAGAGCACAGAGGATGATATGCTTATAGATATACATATAGATACATG
GAGATGAAGATGAAGGGTCAATTAATTGCATTTCATTTCCTTTGTGCTCTCTAGACTTCTGTCATC
CAC

Pre-miR156E



TGCTGTTGACAGAAGATAGAGAGCACAGATGGTGAAATGCATGGAAGGCAATAGCATCTCATTC
CTTTGTGCTCTTTATTCTTCTTGTCACCATCACC

Pre-miR156H

TCATGTTGACAGAAGAGAGAGAGCACAACCCGGGAATGGTGAAAAGAGAGTCTTTGCCTTTGTT
GGGAGTGTGCTCTCCCTTCTTCTGTCATCATCAC

Pre-miR156K

Figure 5.2 Sequence and predicted stem loop diagrams of pea precursor *miRNA156*
The Medicago miRNA precursor sequences in miRBase (http://www.mirbase.org/cgi-bin/query.pl?terms=mtr-miR156&submit=Search; (Kozomara & Griffiths-Jones, 2011, , 2014) were used with tBLASTn to find matching *miR156* sequences in pea. There were no sequences found for *miR156d, miR156f, miR156g, miR156i* and *miR156j* (As shown in Table 5.3). The predicted mature miRNA is shaded in pink and the complementary duplex sequence in blue. Diagrammatic predictions of the miRNA secondary stem loop structures are shown underneath each sequence determined using the RNAfold online server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi; (Lorenz *et al.*, 2011).

5.3.2 The expression patterns of miR156 in pea

In several species, the shift from the juvenile to the adult phase of plant development is thought to coincide with a decline in miR156 and the associated increase in *SPL* expression (Wu *et al.*, 2009). To provide an initial indication of whether miR156 may follow a similar pattern during development in pea, the transcript abundance of miR156 precursor sequences were examined in wild-type plants (NGB5839) across a developmental series (Figure 5.3).

Since the *miRNA156a* precursor sequence originates from *SPL20* this was excluded from analysis and *miRNA156b* was unable to be amplified, so only the expression patterns of *miRNA156c*, *e*, *h* and *k* are shown (Figure 5.3). Wild-type pea plants commit to flowering by two weeks after sowing in long days (Hecht *et al.*, 2011), suggesting that a shift from the juvenile to the adult vegetative phase and any associated decline in miR156 level would most likely happen by this point. *MiR156h* did not show any expression in leaves or apex, and *miR156c*, *e* and *k* did not show the declining pattern of expression expected from studies other species, suggesting that these precursors are unlikely to contribute to declining miR156 levels and therefore may not be involved in vegetative phase change in pea (Figure 5.3). This result is perhaps not surprising given that it is a different miR156 precursor, *miR156d*, which has been shown to affect phase change in overexpression experiments in Medicago and Lotus. This specific precursor has not yet been identified in pea (Aung *et al.*, 2014; Wang *et al.*, 2015).

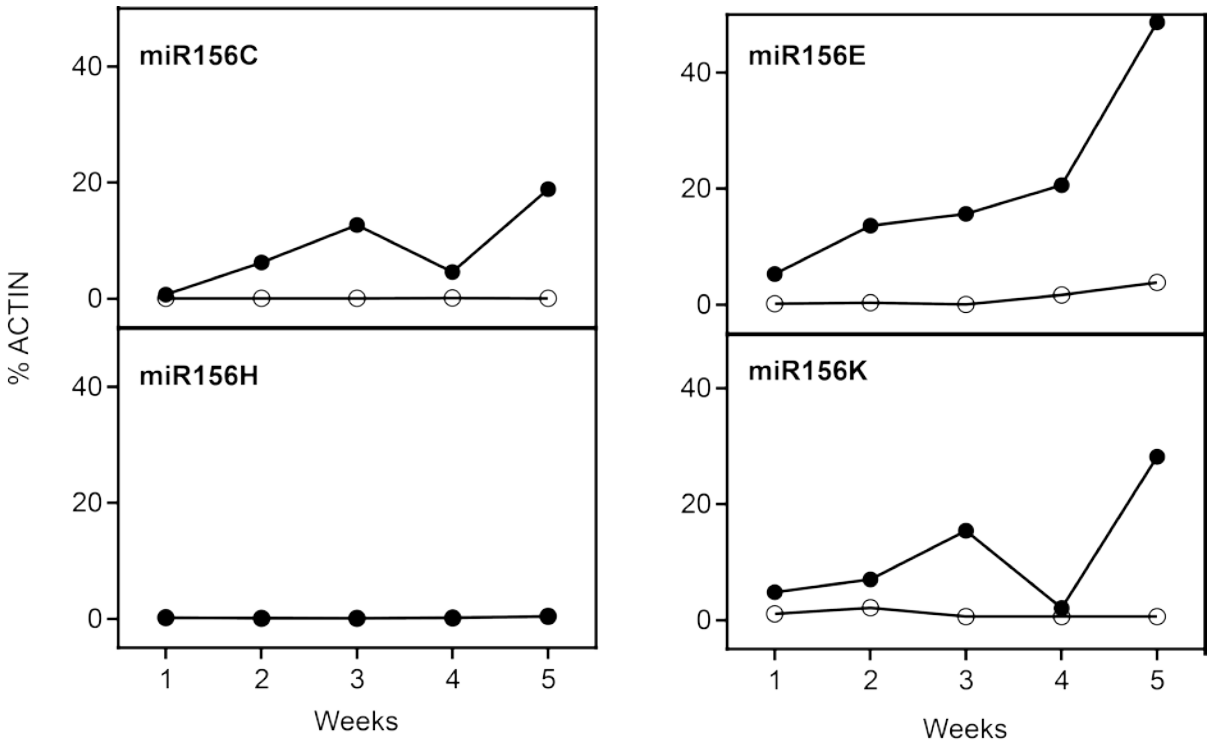


Figure 5.3 Expression patterns of specific pre-miRNA156 transcripts in pea.
Expression of pre-miRNA156 transcripts during development from sowing, over the first five weeks of growth. Relative transcript levels were determined in dissected shoot apices or the uppermost fully expanded leaf during development in long days (apex = open circles and leaf = closed circles). Values have been normalised to the transcript level of the *ACTIN* gene and represent a single biological replicates, each consisting of pooled material from two plants. A second biological replicate was not completed, for the reason that these results showed that these miR156 precursors are not regulated in a manner consistent with a role in vegetative phase change.

5.3.3 The SPL gene family in Medicago and pea

Previously reported Arabidopsis and Medicago SPL sequences from (Cardon *et al.*, 1999; Guo *et al.*, 2008; Preston & Hileman, 2013) were used in BLASTp searches of pea and Medicago protein databases (see online resources in Chapter 2). These sequences were also used for BLASTp back to the original species to confirm there were no other pea or Medicago *SPL* genes missing from the initial searches. As described above, members of the SPL transcription factor family are characterised by a highly conserved SBP domain of ~79 amino acid residues in length (Yamasaki *et al.*, 2004) and on this basis 20 SPLs in pea and 22 in Medicago were identified. Details of these sequences are presented in Table 5.4.

In cases where the genes identified were not annotated, or where alignments suggested that the annotation provided in online genome databases was incorrect, protein sequences were inferred from transcript sequences and from genome sequence based on alignments between species. Most instances of incorrect annotation involved unspliced intron sequence, short sequence duplications or sections missing from the start/end of the gene. Genomic scaffolds for pea SPLs were also obtained where available from the in-progress pea genome sequencing project (Alves-Carvalho *et al.*, 2015) courtesy of Dr G. Aubert, and used for annotations of gene structure.

Table 5.4 Sequence details for SPL proteins used for alignments and phylogenetic analyses

Species	Gene Name	Accession Number (GenBank)	Locus Number (Phytozome / PsCameor)	Reference(s)
Arabidopsis thaliana	SPL1	NP_850468	AT2G47070	(Cardon *et al.*, 1999; Guo *et al.*, 2008; Preston & Hileman, 2013)
	SPL2	NP_851122	AT5G43270	
	SPL3	NP_565771	AT2G33810	
	SPL4	NP_175723	AT1G53160	
	SPL5	NP_188145	AT3G15270	
	SPL6	NP_177077	AT1G69170	
	SPL7	NP_001190333	AT5G18830	
	SPL8	NP_683267	AT1G02065	
	SPL9	NP_181749	AT2G42200	
	SPL10	NP_001031096	AT1G27370	
	SPL11	NP_001077603	AT1G27360	
	SPL12	NP_191562	AT3G60030	
	SPL13A	NP_851161	AT5G50570	
	SPL13B	NP_568740	AT5G50670	
	SPL14	NP_173522	AT1G20980	
	SPL15	NP_191351	AT3G57920	
	SPL16	NP_177784	AT1G76580	
Medicago truncatula	SPL1A	XP_003626036	Medtr7g110320	(Preston & Hileman, 2013) This study
	SPL1B	XP_013463701	Medtr2g046550	
	SPL2A	XP_003601767	Medtr3g085180	
	SPL2B	XP_013446576	Medtr8g080670	
	SPL2C	XP_013446577	Medtr8g080680	
	SPL2D	XP_013446578	Medtr8g080690	
	SPL3A	XP_003593617	Medtr2g014200	
	SPL3B	XP_013456994	Medtr4g088555	
	SPL3C	XP_01344549	Medtr8g463140	
	SPL3D	XP_013464662	Medtr2g078770	
	SPL6A	XP_003614226	Medtr5g046670	
	SPL6B	XP_013458014	Medtr4g109770	
	SPL6C	XP_013464103	Medtr2g461920	
	SPL7	XP_003594035	Medtr2g020620	
	SPL8	XP_003626693	Medtr8g005960	
	SPL9A	XP_013467649	Medtr1g053715	
	SPL9B	XP_003625236	Medtr7g092930	
	SPL13A	XP_003602795	Medtr3g099080	
	SPL13B	XP_013446991	Medtr8g096780	
	SPL13C	XP_013447951	Medtr7g028740	
	SPL14	XP_003589683	Medtr1g035010	
	SPL20	XP_013448322	Medtr7g444860	

Pisum sativum	SPL1A	-	PsCam048790	This study
	SPL1B	-	PsCam048218	
	SPL2A	-	PsCam037189	
	SPL2B	-	PsCam037577	
	SPL3A	-	PsCam033864	
	SPL3B	-	PsCam017682	
	SPL3C	-	PsCam038909	
	SPL3D	-	PsCam047017	
	SPL6A	-	PsCam033315	
	SPL6B	-	PsCam036737	
	SPL6C	-	PsCam025564	
	SPL7	-	PsCam057199	
	SPL8	-	PsCam001061	
	SPL9A	-	PsCam039166	
	SPL9B	-	PsCam037965	
	SPL13A	-	PsCam046016	
	SPL13B	-	PsCam055995	
	SPL13C	-	PsCam012994	
	SPL14	-	PsCam048786	
	SPL20	-	PsCam037459	

In order to investigate the evolutionary relationship between pea and Arabidopsis *SPL* genes, phylogenetic analysis was performed using Arabidopsis, pea and Medicago SBP domain nucleotide sequences. The results showed that *SPL* genes from these species clustered into nine clades (Table 5.5; Figure 5.4). The grouping of Arabidopsis genes in this analysis was consistent with previous phylogenetic trees for that species (Preston & Hileman, 2013) and each Arabidopsis subclade contained one or more legume representatives (Table 5.5). The results in Figure 5.4 also show clear evidence for the expansion of the *SPL* family in legume evolution, with multiple subclades showing duplication events for both legume species. *Medicago* also shows a triplication event in clade 2, which is not present in pea, suggesting a relatively recent origin after the divergence of these two lineages. Legumes also appear to have an additional *SPL* gene that does not correspond to any Arabidopsis *SPL* gene and has been given the unique designation as SPL20. To provide consistency in nomenclature for pea and *Medicago*, the other *SPL* genes have been named (or renamed) according to the closest *Arabidopsis* ortholog in the subclade (Table 5.4; Figure 5.4**).**

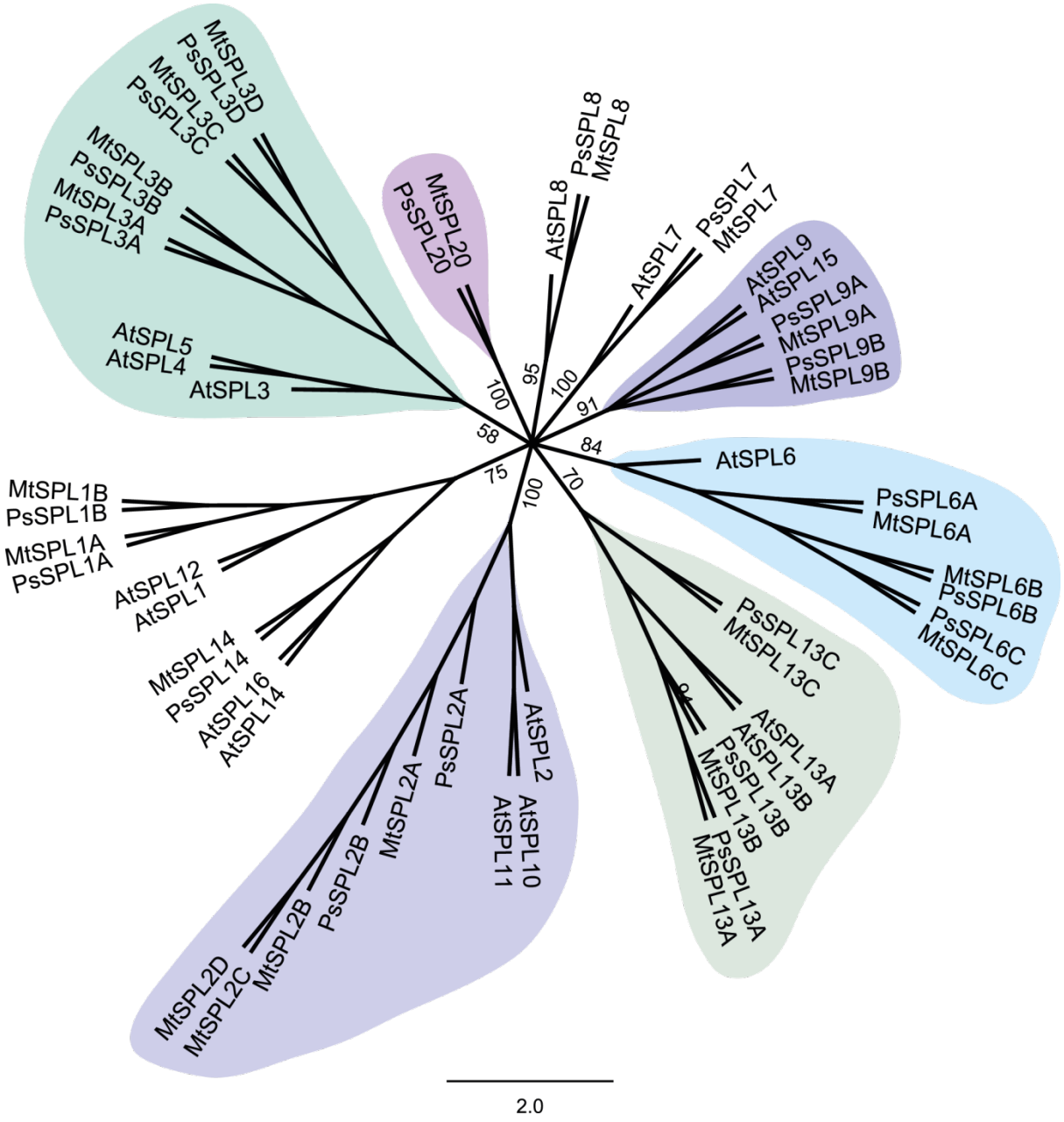


Figure 5.4 SPL phylogenetic tree
A radial neighbour joining tree preformed on the alignment of nucleotide sequences from the SBP domain only in *Arabidopsis thaliana* (At), *Medicago truncatulata* (Mt) and *Pisum sativum* (Ps). The clades with miRNA156 binding sites are shaded in colour. Bootstrap values obtained from 10,000 trees are indicated for each clade. Sequence information can be found in Table 5.4.

Table 5.5 SPL clades in Arabidopsis with the number of genes within each clade for Medicago and pea.

Clade	Known Arabidopsis SPLs in each clade	Clades from Preston & Hileman (2013)	*Arabidopsis thaliana* SPLs	*Medicago* SPLs	*Pea* SPLs
1	1,12, 14, 16	II	4	3	3
2	2,10,11	V	3	4	2
3	3,4,5	VI	3	4	4
4	6	IV	1	3	3
5	7	I	1	1	1
6	8	III	1	1	1
7	9,15	VIII	2	2	2
8	13A,B	VII	2	3	3
9	-	-	0	1	1

5.3.4 SPL *gene structure and miR156 binding sites*

Annotation of gene structure for each of the pea *SPL* genes revealed a diverse genomic organisation among this family of genes (Figure 5.5). Not only are the sizes of the introns and exons highly variable, but also the number of introns per gene varies from one to nine. The similarity of gene structures was consistent with groupings defined by phylogenetic analysis of the SBP domain alone, with all members of a subclades having a similar genomic structure. The SBP domain (shaded red) is coded by the first and second exons and interrupted by an intron in all 20 genes, except for *SPL14* (Figure 5.5).

In Arabidopsis, miR156 binding sites are found in transcripts of 11 *SPL* genes and most of these have been shown to regulate developmental transitions (Chen et al., 2010; Huijser and Schmid, 2011; Xu et al., 2016). We searched the pea *SPL* sequences for the unique miR156 motif and found that all pea *SPL* genes had a miR156 binding site (shaded yellow in Figure 5.5), except for *SPL1A*, *SPL1B*, *SPL7*, *SPL8* and *SPL14*. As in *Arabidopsis SPL3*, *SPL4* and *SPL5*, the miR156 binding sites in pea *SPL3A*, *SPL3B*, *SPL3C* & *SPL3D* are uniquely located in the 3'UTR region. In contrast miR156 binding sites in all other *SPL* genes are located in the final exon of the coding region (Figure 5.5). These results suggest that miR156-mediated regulation of SPLs is likely to be conserved between pea and Arabidopsis.

The Medicago *SPL20* gene is unique because it also appears to give rise to the *miR156a* precursor transcript. This putative transcript is unusually large (460bp) and spans intron 2 in *SPL20* (Table 5.3). Interestingly, this is the only pea/Medicago *SPL* that does not clearly correspond to any Arabidopsis *SPL* (Figure 5.4; Table 5.5). This suggests that either the Medicago *miR156a* precursor sequence is incorrect or that the legume SPL20 has a novel function in that it is both regulated by miR156 and produces miR156 precursor sequences. The latter seems unlikely, but without functional analysis of *miR156a* and without understanding the specific *miR156* that regulate particular *SPLs* in Medicago and pea, this will remain unresolved.

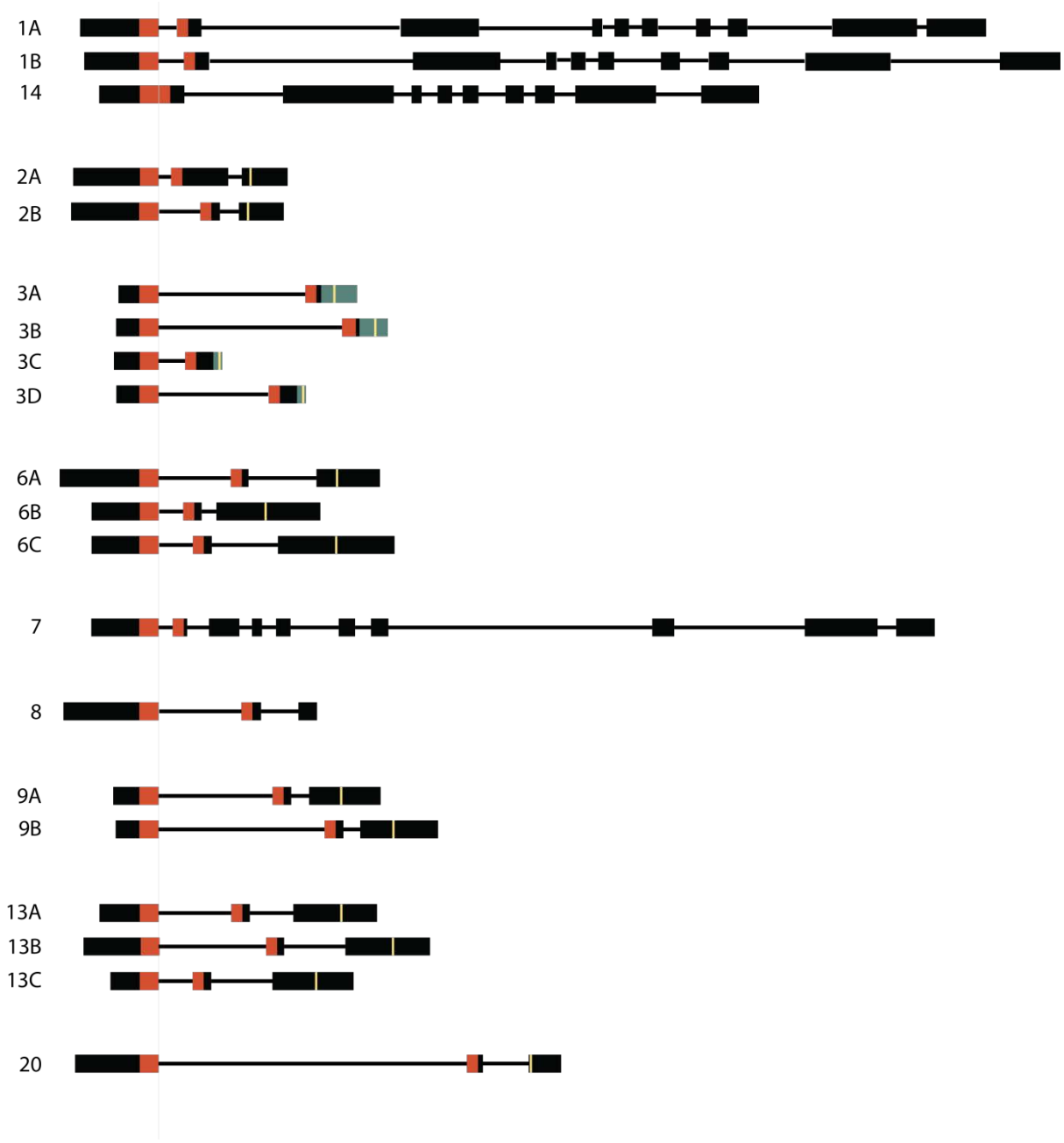


Figure 5.5 The genomic sequence structure of the *SPL* gene family in pea
Scaled schematic representation of the exon/intron structure of the 20 *SPL* genes in pea. The genes are numbered and grouped in line with the different clades of the phylogenetic tree in Figure 5.1. Exons are represented by boxes, introns by horizontal lines between the exons. Untranslated 3' regions are represented in grey. The SBP domain is shaded red and the miR156 binding site is shaded in yellow.

The SBP domains in pea SPL proteins were also examined in more detail. Sequence analysis of the 20 pea SBP domains revealed that they all contain the two conserved zinc-binding sites (so-called Zn1 and Zn2) that each consist of eight cysteine (C) or histidine (H) residues essential for DNA binding, and the conserved nuclear localisation signal (NLS) in the C-terminus region that is required for import into the nucleus (Figure 5.6). These results show that the pea *SPL* proteins are likely to have the ability to be imported into the nucleus and bind in a sequence specific mode to DNA (Yamasaki *et al.*, 2004; Birkenbihl *et al.*, 2005).

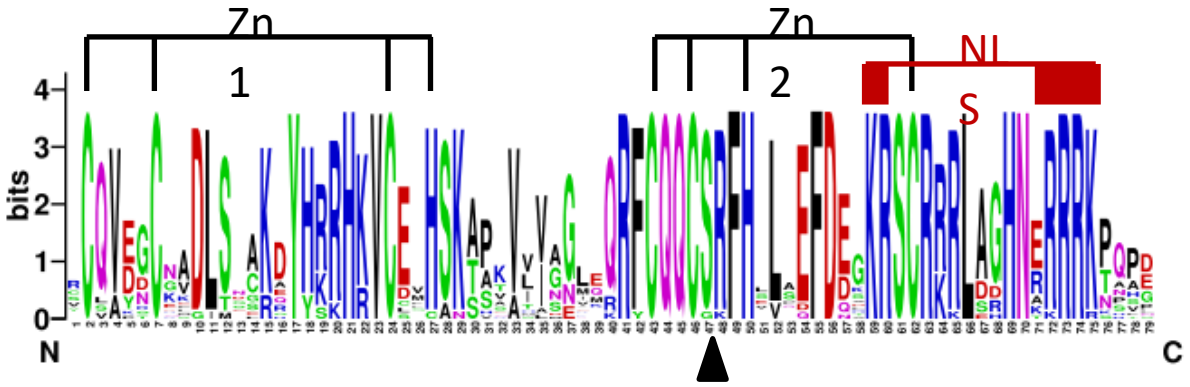


Figure 5.6 Sequence conservation in the SBP-domain for the 20 pea SPLs.
The overall height of each stack indicates the sequence conservation at that position (measured in bits), whereas the height of symbols within the stack reflects the relative frequency of the corresponding amino acid. The two conserved Zn-finger structures and the NLS are indicated. The black triangle between residues 47 and 48 refers to the intron splicing site. (http://weblogo.berkeley.edu/logo.cgi)

5.3.5 Expression patterns of pea SPL *genes*

Finally, developmental variation in expression of *SPL* genes was examined in the same WT developmental series used for miR156 precursors (shown above in Figure 5.3). This analysis was restricted to those *SPL* genes with miR156 binding sites, as these were considered to be the only ones likely to be relevant for a miR156-dependent phase change mechanism. As mentioned, under the conditions of this experiment, *FTb2* induction and floral commitment occurs in WT plants at around two weeks after sowing, and flower buds are visible in the apex by four weeks (Hecht *et al.*, 2011), indicating that both vegetative and reproductive phase change have presumably occurred by this point.

The results in Figure 5.5 show that there was no significant expression of *SPL2B, SPL6A, SPL6C, SPL9A* & *SPL13C* genes in either expanded leaves or apex tissue, so these were excluded from further analysis. For those *SPLs* genes that did show detectable expression, only *SPL3B, SPL3C* & *SPL9B* were expressed in leaf tissue, suggesting a possible role for these genes in leaf development. *SPL3B* and *SPL9B* presented highest expression in the youngest fully expanded leaves of one-week-old plants, whereas *SPL3C* showed increasing expression, being highest in five-week-old plants (Figure 5.7).

Expression of *SPL2A, SPL3A, SPL6B, SPL9B, SPL13A, SPL13B* and *SPL20* all increase in the apex during development (Figure 5.7). The sharpest increase for all genes occurs between 2 and 3 weeks, suggesting that this could be an important stage at which miR156 levels decrease, allowing *SPL* expression to increase. Only *SPL3A* & *SPL3C* continue to increase in expression after 3 weeks, suggesting a possible role later in plant development. As shown in the recent Arabidopsis study, *AtSPL3, SPL4* and *SPL5* do not affect vegetative phase change, but rather, have a later role in the floral meristem identity transition (Xu, Hu, *et al.*, 2016). This may also be the case in pea, considering that the sharpest increase in expression for the *SPL3A* and *SPL3B* genes occurs between weeks 4 and 5 (Figure 5.7).

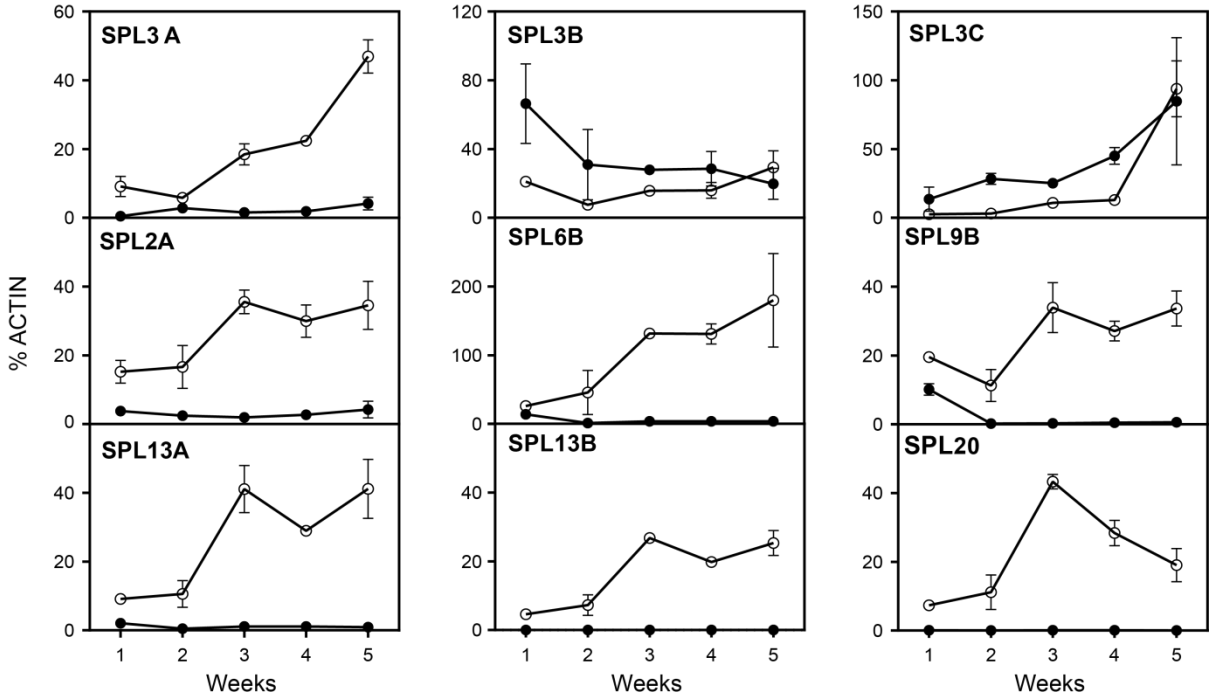


Figure 5.7 Developmental changes in expression of *SPL* genes in pea.
Expression of *SPL* genes during development from sowing, over the first five weeks of growth. Relative transcript levels were determined in dissected shoot apices or the uppermost fully expanded leaf during development in long days (apex = open circles and leaf = closed circles). Values have been normalised to the transcript level of the *ACTIN* gene and the error bars represent mean ± the range for n=2 biological replicates, each consisting of pooled material from two plants.

5.4 Discussion

The deeply conserved miR156 and its targets, the *SPL* gene family, have been implicated in the control of vegetative phase change in a number of species. Across all species studied to date, miR156 expression levels are high in the early stages of plant development and decline with age. Conversely, *SPL* expression increases with age. This chapter has focussed on identifying and isolating the miR156 and *SPL* gene families in pea and examining their expression patterns in different tissues. This work is an important first step in testing whether these components have any function in pea. In future this will help determine whether the phase change mechanism may also be conserved in pea, and reveal which developmental traits, if any, are manifestations of phase change.

Sequence analyses of miR156 and *SPL* genes in pea reveal conservation of gene structure and major gene features when compared with other plant species. These include the miR156 binding site, found in approximately three-quarters of the pea *SPL* genes. On this basis alone it seems likely that the miR156-SPL module is conserved in pea and functions in a similar role with PsmiR156 potentially targeting a subset of the SPL transcription factors. The pea miR156 precursor sequences present all the features observed in miRNAs in general, including the predicted fold-back structure (stem loop) and the duplex containing both the mature miRNA and complementary sequence. A possible additional miR156, previously undiscovered in Medicago, *miR156k*, brings the total miR156 genes in the family to 11 in both pea and Medicago, one more than Arabidopsis, suggesting the number of orthologs has remained largely conserved throughout evolution and adds further weight to the suggestion that miR156 family has more ancient origins compared with other miRNA families (Axtell & Bowman, 2008).

The comprehensive analysis of the *SPL* gene family presented here in pea and Medicago is the first detailed characterization of this family in legumes, apart from one very recent study in the warm season legume, soybean (Tripathi *et al.*, 2017). Results show that pea and Medicago *SPL* genes are similar in structure to the previously described Arabidopsis genes (Cardon *et al.*, 1999; Guo *et al.*, 2008) and with the exception of one gene, *SPL20*, classify into the same major groupings in phylogenetic analysis (Table 5.5; Figure 5.4). However, this analysis is based on the nucleotide sequence of the SBP domain only, in line with phylogenies from other species (Guo *et al.*, 2008; Preston & Hileman, 2013). It is not clear whether *SPL20* may fall into one

of the other miR156 targeted clades if the full SPL sequence was used in phylogenetic analysis, but it is the only SPL without a clear Arabidopsis counterpart in this analysis. In any case, the pea and Medicago *SPL20* genes show general sequence similarity to all other putatively miR156-targeted *SPL* genes, with three exons and the miR156 binding site in the third exon, so there is nothing to suggest that SPL20 is likely to be different in action or function.

MiRNAs can be challenging molecules to amplify because the miRNA precursor consists of a short stable hairpin (stem-loop) and the mature miRNA is approximately 20-22nt, about the length of a standard PCR primer (Voinnet, 2009). In addition, mature miRNAs from the same family, such as miR156, are extremely similar in nucleotide composition, with some being identical. Since the mature miRNA is the key regulatory component, and in some cases it has been found that multiple mature miRNAs can be produced from same precursor sequence with different biological functions (Marco *et al.*, 2012), understanding the expression level of the mature miRNA is also very important. Regulation of miR156 appears to occur both at the transcriptional level (e.g. by sugar) (Yang *et al.*, 2010, , 2013), and during processing of the mature miRNA, as in the example of regulation by temperature changes (Kim *et al.*, 2016). These complexities mean that it is useful to look at expression of both precursor sequence and mature miRNAs, especially when attempting to understand expression differences in miRNAs that may occur in response to genetic or environmental changes. This study was restricted to expression of the miRNA precursor sequences, rather than the mature miRNA sequence. This was due to the ease of designing standard primers to identify individual miRNA members from the pea 156 family, and to provide initial observations of expression during development in WT plants. Further research into miR156 expression in pea should include an attempt to look at expression of both pre-miRNA and mature miR156 expression by using a method such as Stem Loop PCR (Chen, 2005; Varkonyi-Gasic *et al.*, 2007; Schmittgen *et al.*, 2008).

As yet, the specific miR156(s) that may be implicated in vegetative phase change in pea have not been uncovered. In the incomplete list of genomic sequences obtained here, none had the declining expression during development expected from studies in other species. Thus, in future it will be of significant interest to isolate and check expression patterns of the miR156 sequences that are missing, namely *miR156b, miR156d, miR156i, miR156j* and *miR156f* and *miR156g*. Interestingly, overexpression of alfalfa (*Medicago sativa*) *miR156d* (Aung *et al.*, 2014) and the corresponding *Lotus miR156d* (Wang *et al.*, 2015), both resulted in a phenotype of delayed flowering and increased branching, suggesting a possible role in phase change in

these species. Therefore, *miR156d* in pea would be worth investigating first, but until the whole pea genome is available, this will remain problematic.

The intention behind measuring *SPL* gene expression was to observe where and when they were expressed during pea plant development, with the idea that this might provide preliminary insight to which *SPLs* may be involved in phase change in pea and when the transition from juvenile to adult vegetative phase may occur. Among the *SPL* genes containing miR156 binding sites and expressed in the apex, several were upregulated between 2-3 weeks. This time frame coincides with the competence to flower in the apex (Hecht *et al.*, 2011), and the possible transition from the juvenile vegetative to the adult vegetative phase in pea. If this is the case, then the decline in miR156 might actually occur earlier, suggesting that the most of the time points used in the expression analysis are too late to be meaningful and in future, similar expression experiments should include regular time points within the first 14 days, starting at day 1. An additional option to increase our understanding of exactly where vegetative phase change may be occurring, would be to also look at the expression levels of miR172. If the Arabidopsis mechanism is conserved in pea, this should increase over development, as miR156 decreases (Wu *et al.*, 2009).

The speculations based on the results of these preliminary miR156 and *SPL* experiments, can only be definitively tested with the identification of mutants in these gene families. It will certainly be intriguing to determine the function of the various *SPLs* in pea, particularly the ones that may be involved in phase change. Perhaps the best option in this system would be to request TILLING mutants for a specific group of genes. It seems possible that functional redundancy within specific clades might obscure phenotypes in certain single *SPL* mutants, and require the creation of double and triple mutants, making the process of analysis more complex overall. Transgenic manipulation or gene editing could in principle also be used to alter the function of one or multiple *SPL* genes, but each of these presents significant technical challenges in pea where regeneration is relatively difficult. In conclusion, the preliminary investigation into the miRNA156-SPL module in pea has provided some interesting results and insights, which will form the foundation for future research into these important gene families in pea.

Chapter 6

General Discussion

6.1 Summary of main findings

The timing and duration of the different developmental phases during plant ontogeny are crucial for a successful life cycle. In particular, the shift from the juvenile vegetative phase to the adult vegetative phase is a signal for the plant that maturity has been attained and reproduction can occur. The focus of this research has been to investigate the morphological changes that might constitute vegetative phase change in pea, and begin to probe the underlying molecular mechanisms that may control this process through the use of mutants with an apparent acceleration of vegetative development, and investigation of the gene families with a possible role in this process.

The results of this research show that if vegetative phase change exists in pea it is not obvious or discrete. During pea shoot development there is a clearly apparent series of changes in leaf morphology, specifically in the number of leaflets per node. Superficially there appear to be clear stages marked by an increase in the number of leaflets per leaf, which changes from two to four to six leaflets at specific times during development. However, underlying this progression is in fact a more gradual increase in vegetative complexity and the number of leaflets seems more likely to be simply a reflection of increasing resources and a higher threshold required to make additional leaflets. This makes the time frame over which vegetative phase change occurs difficult to determine based simply on leaf change alone.

However, the results from the expression experiment for *SPLs* have provided some additional clues as to when this transition might occur in pea. Given the conservation in sequence structure and the miR156 binding site in many *SPLs*, it is highly likely that the major regulatory mechanism controlling phase change, namely the miR156-SPL module, is conserved in pea. The results in this thesis show that *SPLs* start to increase in expression in the apex when the plants are around 2-3 weeks old. In conjunction with our understanding of flowering time, this suggests that vegetative phase change occurs before 3 weeks of age and also before any increase from two to four leaflets. Thus, the juvenile phase in pea may well be short and the transition to the adult phase not marked by any obvious changes in vegetative morphology.

Nevertheless, the fact that pea vegetative morphology does increase in complexity during ontogeny does suggest a connection to age related factors or processes. Whether these represent a specific developmental pathway or more general changes in energy balance is not clear. However, the four non-allelic mutants used in this research, *aero1*, *aero2*, *apc1* and *apc2* all accelerate the timing of this process, indicating the presence of genes that can control the leaf complexity trait and the rate at which it changes during plant development. In *aero2*, *apc1* and *apc2*, the acceleration in leaf change occurs without affecting the subsequent timing of flowering, confirming that these processes can be uncoupled. When double and triple mutants were generated, the effect on leaf change was additive, suggesting this process is controlled by multiple pathways that converge on the same downstream targets or target processes.

The identification of the specific loci disrupted in *aero1* and *apc1* has provided unexpected but intriguing new information and indicates that these loci may have quite distinct roles, consistent with their additive effects. The candidate genes for the *AERO1* locus were investigated and SNPs were identified in the coding sequence of CLF for two different *aero1* mutant alleles. A SNP affecting a highly conserved amino acid in the CXC domain co-segregated with the *aero1-1* mutant phenotype and a single base pair deletion in the post SET domain sequence changed the final STOP codon position in the second allele, *aero1-10*. Based on the strength of this evidence, the *AERO1* locus is identified as *CLF*, a known epigenetic regulator of gene expression that operates in the Polycomb Repressive Complex 2 (Schubert *et al.*, 2006). The second locus, *APC1*, proved more difficult to identify using the classical mapping population, marker design and candidate gene approach. However, when RNAseq data became available for the *apc1* mutant, an investigation of the locus region revealed a SNP in the conserved AAA domain of FtsH11 that co-segregated perfectly with the *apc1* phenotype. Without a second allele, the *APC1* results remain provisional at this stage, but if correct then APC1 belongs to the FtsH family of proteins that operate in large membrane bound complexes involved in protein quality control, energy production and transport (Yu *et al.*, 2004).

A detailed phenotypic examination of three of the mutants, including the creation of double and triple mutant phenotypes, indicated that they control multiple aspects of plant development, not just leaf change, in line with the putative functions of *AERO1/CLF* and *APC1/ FtsH11* as broad regulators of multiple plant processes. The most interesting results were seen when mutations were combined. Although floral architecture abnormalities were not seen in the single mutants of *aero1* and *aero2*, when combined with *apc1*, they enhanced the floral defects

seen in *apc1*. The same occurred with pod defects. Only *aero2* exhibited pod abnormalities in the single mutants, but when *aero1* and *apc1* were combined, the pod defects increased in severity to the point of creating complete sterility in both the *aero1 apc1* double and the triple mutants. This provides evidence that *APC1*, *AERO1* and *AERO2* function to control major processes of plant development including leaf, floral and pod development, organ positioning and the timing of developmental programs. Therefore, these loci may have either overlapping or partially redundant functions or independent functions that feed into similar downstream targets that control specific biological processes.

6.2 Final thoughts and future directions

The wild-type pea inflorescence structure is a compound raceme, with the main shoot apex that is converted into a primary inflorescence (I1) when flowering is induced. The pea I1 bears compound leaves that increase in complexity during the ageing process and flowering is only distinguishable by the production of secondary inflorescences (I2) (Singer *et al.*, 1999). A question posed by this research is whether vegetative phase change, which is the age related signal for the shoot apex to convert into the I1, can be distinguished by other morphological traits such as compound leaf development.

The expression results of the upregulation of SPLs at around 2-3 weeks suggests that the shift from juvenile to adult in pea occurs very early in plant development. This raises the possibility that the juvenile phase may have already been completed prior to germination. In Arabidopsis only the two cotyledons are set in the embryo before germination and the first two leaf primordia only become apparent 96 hours after imbibition (Irish & Sussex, 1992). Whereas, in pea, during embryogenesis and seed formation, the shoot apex has already committed to the development of 5-6 true leaves, so the seedling is already partly developed before germination even occurs (Blixt, 1974). The transition from the juvenile to the adult form would certainly have already occurred prior to germination for those genotypes that flower very early, such as *sn* and *lf* (Wiltshire *et al.*, 1994). If the first phase transition has occurred before germination, then this may be why it is difficult to discern any morphological changes in the developing stem to signal vegetative phase change after germination. In addition, this could also imply that the mutants studied in this research are simply shifted in the timing of the compound leaf

developmental sequence as part of the I1, rather than affecting the timing of vegetative phase change itself.

Assuming that the identities of *AERO1* and *APC* genes can be confirmed and the tentative conclusions made here are correct, then it becomes necessary to explore and explain how they interact to affect some of the same biological processes, particularly considering that CLF & FtsH11 are vastly different in their primary molecular roles. There are a number of scenarios that could explain this. One possible explanation is that CLF positively regulates FtsH11 epigenetically, so that when CLF is absent, *FtsH11* expression is reduced, causing a mutant phenotype similar to the *ftsh11* mutant. However, in general, the PRC2 complex is a repressor of gene expression (Köhler & Grossniklaus, 2002), so removing the effect of CLF would potentially increase the function of *FtsH11*, not decrease it, as we expect to be the case in the *ftsh11* mutant.

An alternative explanation, and likely more plausible, is that both FtsH11 and CLF operate to affect similar targets, but in vastly different ways. For instance, it is known that FtsHs function as proteases, removing excess or damaged proteins (Janska *et al.*, 2010; Wagner *et al.*, 2012). When this function is disturbed in Arabidopsis, as studied in *ftsh4* mutants, levels of peroxidases increase causing oxidative stress, which in turn affects auxin homeostasis in the plant. In addition, several auxin transport genes were significantly downregulated in *ftsh4* mutants (Pasternak *et al.*, 2005; Zhang *et al.*, 2014). When a study of H3K27me3 changes in *clf* mutants was undertaken by (Lafos *et al.*, 2011), the majority of auxin biosynthesis and transport genes were also found to have altered levels of expression when CLF was non-functional, suggesting a major role for CLF in maintaining auxin homeostasis as well. Thus, it is possible that CLF and FtsH11, although they have very different modes of action, could affect the same downstream targets that cause the similar phenotype in both mutants.

The pleiotropic nature of the mutant phenotypes are interesting and raise many questions about what downstream processes might be affected by a disruption in function of FtsH11/APC1, APC2, CLF/AERO1 and AERO2. A possible explanation for their nature is that these loci are primarily involved in regulating processes that occur in the plant meristem. Lateral organ formation, phyllotaxy and the floral meristem development are all processes that are determined in the shoot apical meristem (SAM). One possibility is that all four genes affect the size, shape or cellular structure of the apex. We examined this in a preliminary study, and found

significant changes to the shape of the apex and the developing vegetative organs in the single mutants, a result that does add some support to this theory. It will be interesting in future to extend these experiments to determine whether these changes are more severe in double mutants and to characterize how SAM morphology changes over time in WT plants. However, it is important to point out that the fate of immature leaves and floral organ primordia can be altered without changing the fate of the cells growing in the SAM (Sachs, 1969; Battey & Lyndon, 1988; Orkwiszewski & Poethig, 2000), so it is also possible that the four loci investigated are acting on the young developing organs at the shoot apex rather than specifically in the SAM. Our results add support to this interpretation, as the pea mutants had little or no impact on the number of cells in the SAM, but showed changes in the shape of the apex and developing leaf primordia. Because the apex is vital to so many aspects of plant development, it is dynamically regulated by a diverse and complex signalling network consisting of the activities of many genes and plant hormones (Truskina & Vernoux, 2018). Coordination among these activities is a prerequisite for maintaining a properly functioning shoot apex. This could also provide an explanation for the increasing severity among the double and triple mutants. If the precise coordination of the apex is eroded through additional mutations to important regulatory factors, this would likely lead to multiple, severe defects in plant development.

Given that there is evidence for the disruption of the levels and regulation of the hormone auxin in both *ftsh* and *clf* mutants in Arabidopsis (Lafos *et al.*, 2011; Zhang *et al.*, 2014), one simple, broad explanation for the mutant phenotypes could be that they all have an underlying disruption of auxin homeostasis. Many aspects of plant growth and development are regulated by auxin, and in particular, organ initiation and pattern formation largely depend on the spatiotemporal control of auxin levels (Benková *et al.*, 2003; Heisler *et al.*, 2005). Local gradients of auxin mediated by the PIN-FORMED (PIN) auxin transport proteins are required for both determining the position of leaf or floral organ inception along the periphery of the SAM and promoting organogenesis. (Benková *et al.*, 2003; Reinhardt *et al.*, 2003; Fleming, 2005; Heisler *et al.*, 2005). In addition, in species with compound leaf species such as pea, local auxin maxima are required for the regulation of organ shape, size and dissection of leaflets after the leaf primordium has started developing (Barkoulas *et al.*, 2008; Koenig *et al.*, 2009; DeMason *et al.*, 2013). Future research looking at PIN expression, and other auxin related genes in the pea mutants would be interesting to determine whether there was indeed a disruption of auxin homeostasis. It would also be highly informative to determine the spatial

distribution of auxin in the SAM and developing leaf primordia in the mutants with the use of the available DR5 reporter constructs (DeMason & Polowick, 2009).

Despite the various lines of investigation followed in this thesis, it is still difficult to establish any obvious link between the mutant loci and phase change. This is particularly the case after the discovery that *AERO1* may regulate gene expression epigenetically as the ortholog of *AtCLF*, and that *APC1* possibly functions as a transporter and protease. However, the genes involved in control of vegetative phase change must at some point interact with genes that control developing organ primordia in the apex since phase change is manifest in leaves and involves alterations to leaf and shoot developmental programs (Poethig, 2013).

For instance, how do the newly developing leaf primordia know that the plant has transitioned from the juvenile to the adult vegetative phase, and change its morphology accordingly? If this information does not originate in the SAM, as research has suggested (Yang *et al.*, 2011), then the AERO and APC loci either act on the phase change identity signal originating elsewhere that then acts on the developing organs or they control aspects of normal primordium formation that are also affected by genes specifically controlling phase identity. Given the pleiotropic phenotypes of the mutants, the latter idea seems more likely. Interestingly, the recent study in tomato showed that two mutants, *mouse ears* (*me*) and *fasciated* (*fas*), that are involved in the control of the balance between differentiated and undifferentiated cells in the shoot meristem, also had a similar phenotype to a line overexpressing miR156, the key regulator of phase change, providing evidence that these processes are linked (Vendemiatti *et al.*, 2017). It would be interesting to explore this idea further in pea, through looking at the cellular structure of developing leaves over time. Is the leaf change phenotype seen in the pea mutants due to an increased rate of cell division and expansion in specific locations of the developing leaf primordia? Once again, it will be of great interest to generate or identify mutants defective in miR156 expression or their downstream *SPL* targets, to compare their phenotypes with those of the mutants studied here, particularly with respect to the shift in the timing of leaf change.

Given the difficulties in defining vegetative phase change with morphological changes in pea as this research has shown, focussing future work on the master regulators of phase change to answer further questions would be helpful. Finding the correct miR156 and miR172 regulating phase change in pea and developing the methods for quantifying the mature miRNAs would

be extremely useful. Further, checking miR156 levels in the mutants lines would also provide clear evidence as to whether they were regulating phase change on not.

Finally, studies on the juvenile to adult phase transition have significant scientific, agricultural and economic implications. Juvenility has long attracted research interest as a major feature in plant ontogeny, partly for fundamental reasons but also for the practical consequences that arise from alterations to the plant's juvenile period. Understanding the factors that control the timing and duration of the juvenile phase is critical for any crop species to increase their breeding potential, habitat range and commercial value. Decreasing the length of the juvenile phase can shorten the time a plant species takes to flower, producing a crop sooner or increasing viability in areas where growing seasons are shorter. However, this can often come at the expense of reproductive output such as fruit or biomass yield. Conversely, increasing the length of the juvenile phase can also be of benefit in greater biomass production through increased branching and sometimes higher yields, but generation time is lengthened, taking longer for crops to reach maturity. In understanding the genetic control of the juvenile phase and how it is connected to flowering during legume development could provide ways to increase yield, without delaying flowering time. Although not directly related to the phase change associated pathways, the mutant alleles in pea and their identified loci provide further understanding of the genetic control of vegetative traits, particularly changes in leaf complexity during development. The interesting thing about the *apc1* and *apc2* mutants in pea, is that they potentially increase the amount of biomass, without affecting the timing of flowering or seed yield. This may prove to be a useful trait in crop production, particularly if these sorts of varieties were used as green manure or fodder for animals, as peas and many other legumes are. Of further interest would be to look at the FtsH mutants in other legume species, to not only confirm our findings, but also to create possible new varieties that may be of agricultural benefit.

6.3 Concluding Remarks

In conclusion, the results of this thesis provide the framework and first essential results for unravelling the regulation of phase change in pea and provide a foundation for future research in this area. Characterisation of the other two loci AERO2 and APC2, identifying the relevant miR156 and miR172 species in vegetative phase change regulation, along with creation of mutants in key genes, would be the next steps to increasing our knowledge in this area and provide exciting prospects for further scientific discovery.

References

Afgan, E., Baker, D., van den Beek, M., Blankenberg, D., Bouvier, D., Čech, M., Chilton, J., Clements, D., Coraor, N., Eberhard, C., Grüning, B., Guerler, A., Hillman-Jackson, J., Von Kuster, G., Rasche, E., Soranzo, N., Turaga, N., Taylor, J., Nekrutenko, A., & Goecks, J. 2016. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res.* 44(W1):W3–W10.

Akiyama, Y., Ogura, T., & Ito, K. 1994. Involvement of FtsH in Protein Assembly into and through the Membrane. *J. Biol. Chem.* 269(7):5218–5224.

Akiyama, Y., Yoshihisa, T., & Ito, K. 1995. FtsH, a membrane-bound ATPase, forms a complex in the cytoplasmic membrane of Escherichia coli. *J. Biol. Chem.* 270(40):23485–23490.

Allsop, A. 1954. Juvenile Stages of Plants and the Nutritional Status of the Shoot Apex. *Nature*. 173(4413):1032–1035.

Allsop, A. 1967. Heteroblastic Development in Vascular Plants. , in: Advances in Morphogenesis. Academic Press, New York. . Academic Press, New York, pp. 127–171.

Alves-Carvalho, S., Aubert, G., Carrère, S., Cruaud, C., Brochot, A.-L., Jacquin, F., Klein, A., Martin, C., Boucherot, K., Kreplak, J., da Silva, C., Moreau, S., Gamas, P., Wincker, P., Gouzy, J., & Burstin, J. 2015. Full-length *de novo* assembly of RNA-seq data in pea (*Pisum sativum* L.) provides a gene expression atlas and gives insights into root nodulation in this species. *Plant J.* 84(1):1–19.

Arazi, T., Talmor-Neiman, M., Stav, R., Riese, M., Huijser, P., & Baulcombe, D.C. 2005. Cloning and characterization of micro-RNAs from moss: miRNAs in moss. *Plant J.* 43(6):837–848.

Arnold, I. & Langer, T. 2002. Membrane protein degradation by AAA proteases in mitochondria. *Biochim. Biophys. Acta BBA-Mol. Cell Res.* 1592(1):89–96.

Ashby, E. 1948. Studies in the morphogenesis of leaves. *New Phytol.* 47(2):153–176.

Aubert, G., Morin, J., Jacquin, F., Loridon, K., Quillet, M.C., Petit, A., Rameau, C., Lejeune-Hénaut, I., Huguet, T., & Burstin, J. 2006. Functional mapping in pea, as an aid to the candidate gene selection and for investigating synteny with the model legume Medicago truncatula. *Theor. Appl. Genet.* 112(6):1024–1041.

Aukerman, M.J. 2003. Regulation of Flowering Time and Floral Organ Identity by a MicroRNA and Its APETALA2-Like Target Genes. *Plant Cell*. 15(11):2730–2741.

Aukerman, M.J. & Sakai, H. 2003. Regulation of Flowering Time and Floral Organ Identity by a MicroRNA and Its APETALA2-Like Target Genes. *Plant Cell*. 15(11):2730–2741.

Aung, B., Gruber, M.Y., Amyot, L., Omari, K., Bertrand, A., & Hannoufa, A. 2014. MicroRNA156 as a promising tool for alfalfa improvement. *Plant Biotechnol. J.* 13(6):779–790.

Aung, B., Gruber, M.Y., Amyot, L., Omari, K., Bertrand, A., & Hannoufa, A. 2015. Ectopic expression of LjmiR156 delays flowering, enhances shoot branching, and improves forage quality in alfalfa. *Plant Biotechnol. Rep.* 9(6):379–393.

Axtell, M.J. & Bartel, D.P. 2005. Antiquity of MicroRNAs and Their Targets in Land Plants. *Plant Cell Online*. 17(6):1658–1673.

Axtell, M.J. & Bowman, J.L. 2008. Evolution of plant microRNAs and their targets. *Trends Plant Sci.* 13(7):343–349.

Bailey, S., Thompson, E., Nixon, P.J., Horton, P., Mullineaux, C.W., Robinson, C., & Mann, N.H. 2002. A Critical Role for the Var2 FtsH Homologue of *Arabidopsis thaliana* in the Photosystem II Repair Cycle *in Vivo*. *J. Biol. Chem.* 277(3):2006–2011.

Barber, H.N. 1959. Physiological Genetics Of Pisum II. The Genetics of Photoperiodism and Vernalisation. *Heredity*. 13:33–60.

Barkoulas, M., Hay, A., Kougioumoutzi, E., & Tsiantis, M. 2008. A developmental framework for dissected leaf formation in the *Arabidopsis* relative *Cardamine hirsuta*. *Nat. Genet.* (40):1136–1141.

Bartel, D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 116(2):281–297.

Battey, N.H. & Lyndon, R.F. 1988. Determination and differentiation of leaf and petal primordia in Impatiens balsamina. *Ann. Bot.* 61(1):9–16.

Bemer, M. & Grossniklaus, U. 2012. Dynamic regulation of Polycomb group activity during plant development. *Curr. Opin. Plant Biol.* 15(5):523–529.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., & Friml, J. 2003. Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell*. 115(5):591–602.

Bergonzi, S., Albani, M.C., van Themaat, E.V.L., Nordström, K.J., Wang, R., Schneeberger, K., Moerland, P.D., & Coupland, G. 2013. Mechanisms of age-dependent response to winter temperature in perennial flowering of Arabis alpina. *Science*. 340(6136):1094–1097.

Beyer, A. 1997. Sequence analysis of the AAA protein family. *Protein Sci.* 6(10):2043–2058.

Bhattacharyya, M.K., Smith, A.M., Ellis, T.H.N., Hedley, C., & Martin, C. 1990. The Wrinkled-Seed Character of Pea Described by Mendel Is Caused by a Transposon-like Insertion in a Gene Encoding Starch-Branching Enzyme. 60(1):115–122.

Bhogale, S., Mahajan, A.S., Natarajan, B., Rajabhoj, M., Thulasiram, H.V., & Banerjee, A.K. 2014. MicroRNA156: A Potential Graft-Transmissible MicroRNA That Modulates Plant Architecture and Tuberization in Solanum tuberosum ssp. andigena. *Plant Physiol.* 164(2):1011–1027.

Bieniek, M.E. & Millington, W.F. 1967. Differentiation of Lateral Shoots as Thorns in Ulex europaeus. *Am. J. Bot.* 54(1):61.

Bieniossek, C., Niederhauser, B., & Baumann, U.M. 2009. The crystal structure of apo-FtsH reveals domain movements necessary for substrate unfolding and translocation. *Proc. Natl. Acad. Sci.* 106(51):21579–21584.

Birkenbihl, R.P., Jach, G., Saedler, H., & Huijser, P. 2005. Functional Dissection of the Plant-specific SBP-Domain: Overlap of the DNA-binding and Nuclear Localization Domains. *J. Mol. Biol.* 352(3):585–596.

Blixt, S. 1962. Studies in induced mutations in peas. VI Mutations in seed colour, flower colour, maculum colour, pod colour and grey spotting of leaves. *Acta Hort Genet*. 20:95–110.

Blixt, S. 1974. The Pea. , in: Handbook of Genetics. Springer: US. . Springer: US, pp. 181–221.

Bollman, K.M. 2003. HASTY, the Arabidopsis ortholog of exportin 5/MSN5, regulates phase change and morphogenesis. *Development*. 130(8):1493–1504.

Bologna, N.G., Schapire, A.L., & Palatnik, J.F. 2012. Processing of plant microRNA precursors. *Brief. Funct. Genomics*. 12(1):37–45.

Bongard-Pierce, D.K., Evans, M.M.S., & Poethig, R.S. 1996. Heteroblastic features of leaf anatomy in maize and their genetic regulation. *Int. J. Plant Sci.* 157(4):331–340.

Bordat, A., Savois, V., Nicolas, M., Salse, J., Chauveau, A., Bourgeois, M., Potier, J., Houtin, H., Rond, C., & Murat, F. 2011. Translational Genomics in Legumes Allowed Placing In Silico 5460 Unigenes on the Pea Functional Map and Identified Candidate Genes in Pisum sativum L. *G3 Genes Genomes Genet.* 1(2):93–103.

Boureau, L., How-Kit, A., Teyssier, E., Drevensek, S., Rainieri, M., Joubès, J., Stammitti, L., Pribat, A., Bowler, C., Hong, Y., & Gallusci, P. 2016. A CURLY LEAF homologue controls both vegetative and reproductive development of tomato plants. *Plant Mol. Biol.* 90(4–5):485–501.

Cao, D., Li, Y., Wang, J., Nan, H., Wang, Y., Lu, S., Jiang, Q., Li, X., Shi, D., Fang, C., Yuan, X., Zhao, X., Li, X., Liu, B., & Kong, F. 2015. GmmiR156b overexpression delays flowering time in soybean. *Plant Mol. Biol.* 89(4–5):353–363.

Cardon, G., Höhmann, S., Klein, J., Nettesheim, K., Saedler, H., & Huijser, P. 1999. Molecular characterisation of the Arabidopsis SBP-box genes. *Gene*. 237(1):91–104.

Cardon, G., Hohmann, S., Nettesheim, K., Saedler, H., & Huijser, P. 1997. Functional analysis of the *Arabidopsis thaliana* SBP-box gene SPL3: a novel gene involved in the floral transition. *Plant J.* 12(2):367–377.

Chanvivattana, Y. 2004. Interaction of Polycomb-group proteins controlling flowering in Arabidopsis. *Development*. 131(21):5263–5276.

Chaudhury, A., Letham, S., Craig, S., & Dennis, E.S. 1993. amp1-a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. *Plant J.* 4(6):907–916.

Chen, C. 2005. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.* 33(20):e179–e179.

Chen, J., Burke, J.J., Velten, J., & Xin, Z. 2006. FtsH11 protease plays a critical role in Arabidopsis thermotolerance. *Plant J.* 48(1):73–84.

Chen, L.-J., Diao, Z.-Y., Specht, C., & Sung, Z.R. 2009. Molecular Evolution of VEF-Domain-Containing PcG Genes in Plants. *Mol. Plant*. 2(4):738–754.

Chen, M., Xia, X., Zheng, H., Yuan, Z., & Huang, H. 2004. The GAOLAOZHUANGREN2 gene is required for normal glucose response and development of Arabidopsis. *J. Plant Res.* 117(6):473–476.

Chen, X. 2004. A microRNA as a translational repressor of *APETALA2* in Arabidopsis flower development. *Science*. 303:2022–2025.

Chen, X., Zhang, Z., Liu, D., Zhang, K., Li, A., & Mao, L. 2010. SQUAMOSA Promoter-Binding Protein-Like Transcription Factors: Star Players for Plant Growth and Development. *J. Integr. Plant Biol.* 52(11):946–951.

Chen, Z., Gao, X., & Zhang, J. 2015. Alteration of osa-miR156e expression affects rice plant architecture and strigolactones (SLs) pathway. *Plant Cell Rep.* 34(5):767–781.

Chien, J.C. & Sussex, I.M. 1996. Differential regulation of trichome formation on the adaxial and abaxial leaf surfaces by gibberellins and photoperiod in *Arabidopsis thaliana* (L.) Heynh. *Plant Physiol.* 111(4):1321–1328.

Choi, H.-K., Mun, J.-H., Kim, D.-J., Zhu, H., Baek, J.-M., Mudge, J., Roe, B., Ellis, N., Doyle, J., Kiss, G.B., & others. 2004. Estimating genome conservation between crop and model legume species. *Proc. Natl. Acad. Sci.* 101(43):15289–15294.

Chuck, Cigan, A.M., Saeteurn, K., & Hake, S. 2007. The heterochronic maize mutant Corngrass1 results from overexpression of a tandem microRNA. *Nat. Genet.* 39(4):544–549.

Chuck, Meeley, R., Irish, E, Sakai, H., & Hake, S. 2007. The maize *tasselseed4* microRNA controls sex determination and meristem cell fate by targeting *Tasselseed6/indeterminate spikelet1*. *Nat. Genet.* 39:1517–1521.

Clarke, J.H., Tack, D., Findlay, K., Van Montagu, M., & Lijsebettens, M. 1999. The *SERRATE* locus controls the formation of the early juvenile leaves and phase length in *Arabidopsis*. *Plant J.* 20(4):493–501.

Dalmais, M., Schmidt, J., Le Signor, C., Moussy, F., Burstin, J., Savois, V., Aubert, G., Brunaud, V., de Oliveira, Y., Guichard, C., & others. 2008. UTILLdb, a Pisum sativum in silico forward and reverse genetics tool. *Genome Biol.* 9(2):1.

DeMason, D.A., Chetty, V., Barkawi, L.S., Liu, X., & Cohen, J.D. 2013. Unifoliata-Afila interactions in pea leaf morphogenesis. *Am. J. Bot.* 100(3):478–495.

DeMason, D.A. & Polowick, P.L. 2009. Patterns of *DR5::GUS* Expression in Organs of Pea (*Pisum sativum*). *Int. J. Plant Sci.* 170(1):1–11.

Deng, W., Liu, C., Pei, Y., Deng, X., Niu, L., & Cao, X. 2007. Involvement of the Histone Acetyltransferase AtHAC1 in the Regulation of Flowering Time via Repression of FLOWERING LOCUS C in Arabidopsis. *PLANT Physiol.* 143(4):1660–1668.

Dezulian, T., Palatnik, J.F., Huson, D., & Weigel, D. 2005. Conservation and divergence of microRNA families in plants. *Genome Biol*. 6:P13.

Doorenbos, J. 1965. Juvenile and adult phases in woody plants. , in: Differenzierung Und Entwicklung/Differentiation and Development. Springer Berlin Heidelberg. . Springer Berlin Heidelberg, pp. 1222–1235.

Doyle, J.J. 2001. Leguminosae. , in: Encyclopedia of Genetics. Academic Press: San Diego. . Academic Press: San Diego, pp. 1081–1085.

Eriksson, S. 2006. GA4 Is the Active Gibberellin in the Regulation of LEAFY Transcription and Arabidopsis Floral Initiation. *Plant Cell*. 18(9):2172–2181.

Evans, M.M. & Poethig, R.S. 1995. Gibberellins promote vegetative phase change and reproductive maturity in maize. *Plant Physiol.* 108(2):475–487.

Fahlgren, N., Montgomery, T.A., Howell, M.D., Allen, E., Dvorak, S.K., Alexander, A.L., & Carrington, J.C. 2006. Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA Affects Developmental Timing and Patterning in Arabidopsis. *Curr. Biol.* 16(9):939–944.

Feldman, L.J. & Cutter, E.G. 1970. Regulation of Leaf Form in *Centaurea solstitialis* L. I. Leaf Development on Whole plants in Sterile Culture. *Bot. Gaz.* 131(1):31–39.

Ferro, M., Brugière, S., Salvi, D., Seigneurin-Berny, D., Moyet, L., Ramus, C., Miras, S., Mellal, M., Le Gall, S., Kieffer-Jaquinod, S., & others. 2010. AT_CHLORO, a comprehensive chloroplast proteome database with subplastidial localization and curated information on envelope proteins. *Mol. Cell. Proteomics*. 9(6):1063–1084.

Fleming, A.J. 2005. Formation of primordia and phyllotaxy. *Curr. Opin. Plant Biol.* 8(1):53–58.

Foo, E., Bullier, E., Goussot, M., Foucher, F., Rameau, C., & Beveridge, C.A. 2005. The Branching Gene RAMOSUS1 Mediates Interactions among Two Novel Signals and Auxin in Pea. *Plant Cell*. 17(2):464–474.

Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., García, J.A., & Paz-Ares, J. 2007. Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat. Genet.* 39(8):1033–1037.

Franssen, S.U., Shrestha, R.P., Bräutigam, A., Bornberg-Bauer, E., & Weber, A.P. 2011. Comprehensive transcriptome analysis of the highly complex Pisum sativum genome using next generation sequencing. *BMC Genomics*. 12(1):1.

Galinat, W.C. 1966. The corn grass and teopod loci involve phase change. *Maize Genet. Coop. Newsl.* 40:102–103.

Galvao, V.C., Horrer, D., Kuttner, F., & Schmid, M. 2012. Spatial control of flowering by DELLA proteins in Arabidopsis thaliana. *Development*. 139(21):4072–4082.

Gandikota, M., Birkenbihl, R.P., Höhmann, S., Cardon, G.H., Saedler, H., & Huijser, P. 2007. The miRNA156/157 recognition element in the 3' UTR of the Arabidopsis SBP box gene SPL3 prevents early flowering by translational inhibition in seedlings: miRNA regulation of SPL3. *Plant J.* 49(4):683–693.

Gao, R., Austin, R.S., Amyot, L., & Hannoufa, A. 2016. Comparative transcriptome investigation of global gene expression changes caused by miR156 overexpression in Medicago sativa. *BMC Genomics*. 17(1).

Gasciolli, V., Mallory, A.C., Bartel, D.P., & Vaucheret, H. 2005. Partially Redundant Functions of Arabidopsis DICER-like Enzymes and a Role for DCL4 in Producing trans-Acting siRNAs. *Curr. Biol.* 15(16):1494–1500.

Gatsuk, L.E., Smirnova, O.V., Vorontzova, L.I., Zaugolnova, L.B., & Zhukova, L.. 1980. Age States of Plants of Various Growth Forms: A Review. *J. Ecol.* 68:675–696.

Gazzarrini, S., Tsuchiya, Y., Lumba, S., Okamoto, M., & McCourt, P. 2004. The transcription factor FUSCA3 controls developmental timing in Arabidopsis through the hormones gibberellin and abscisic acid. *Dev. Cell*. 7(3):373–385.

Gibala, M., Kicia, M., Sakamoto, W., Gola, E.M., Kubrakiewicz, J., Smakowska, E., & Janska, H. 2009. The lack of mitochondrial AtFtsH4 protease alters Arabidopsis leaf morphology at the late stage of rosette development under short-day photoperiod. *Plant J.* 59(5):685–699.

Gillmor, C.S., Silva-Ortega, C.O., Willmann, M.R., Buendia-Monreal, M., & Poethig, R.S. 2014. The Arabidopsis Mediator CDK8 module genes CCT (MED12) and GCT (MED13) are global regulators of developmental phase transitions. *Development*. 141(23):4580–4589.

Goebel, K. 1889. Ueber die Jugend Zustände der Pflanzen. *Flora*. 72:1–44.

Goebel, K. 1908. Einleitung in die experimentelle morphologie der Pflanzen. *Science*. 28(723):650–651.

Goethe, J.W.V. 1790. Versuch die Metamorphose der Pflanzen zu erklaren. *Ger. Gotha*.

Goldenberg, J.B. 1965. Afila, a new mutation in pea (Pisum sativum L.). *Boletin Genet.* 1:27–31.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E. m., & Coupland, G. 1997. A polycomb-group gene regulates homeotic gene expression in Arabidopsis. *Nature*. 386(6):44–51.

Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., & Rokhsar, D.S. 2012. Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Res.* 40(D1):D1178–D1186.

Gould, K.S. 1993. Leaf heteroblasty in *Pseudopanax crassifolius*: functional significance of leaf morphology and anatomy. *Ann. Bot.* 71:61–70.

Gould, K.S., Young, J.P.W., & Cutter, E.G. 1992. L-System analysis of compound leaf development in Pisum sativum L. *Ann. Bot.* 70(2):189–196.

Graham, P.H. 2003. Legumes: Importance and Constraints to Greater Use. *Plant Physiol.* 131(3):872–877.

Grimbly, P.E. 1977. Tomato silvering, its anatomy and chimerical structure. *J. Hortic. Sci.* 52:469–473.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., & Gascuel, O. 2010. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Syst. Biol.* 59(3):307–321.

Guindon, S. & Gascuel, O. 2003. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Syst. Biol.* 52(5):696–704.

Guo, A.-Y., Zhu, Q.-H., Gu, X., Ge, S., Yang, J., & Luo, J. 2008. Genome-wide identification and evolutionary analysis of the plant specific SBP-box transcription factor family. *Gene*. 418(1–2):1–8.

Gupta, S., Nadarajan, N., & Gupta, D.S. 2014. Legumes in the Omic Era. Springer-Verlag: New York.

Hackett, W.P. 1985. Juvenility, Maturation, and Rejuvenation in Woody Plants. , in: Horticultural Reviews. John Wiley & Sons, Inc.: Hoboken, NJ, USA. . John Wiley & Sons, Inc.: Hoboken, NJ, USA.

Hecht, V., Foucher, F., Ferrándiz, C., Macknight, R., Navarro, C., Morin, J., Vardy, M.E., Ellis, N., Beltrán, J.P., Rameau, C., & others. 2005. Conservation of Arabidopsis flowering genes in model legumes. *Plant Physiol.* 137(4):1420–1434.

Hecht, V., Knowles, C.L., Vander Schoor, J.K., Liew, L.C., Jones, S.E., Lambert, M.J.M., & Weller, J.L. 2007. Pea LATE BLOOMER1 Is a GIGANTEA Ortholog with Roles in Photoperiodic Flowering, Deetiolation, and Transcriptional Regulation of Circadian Clock Gene Homologs. *Plant Physiol.* 144(2):648–661.

Hecht, V., Laurie, R.E., Vander Schoor, J.K., Ridge, S., Knowles, C.L., Liew, L.C., Sussmilch, F.C., Murfet, I.C., Macknight, R.C., & Weller, J.L. 2011. The Pea *GIGAS* Gene Is a *FLOWERING LOCUS T* Homolog

Necessary for Graft-Transmissible Specification of Flowering but Not for Responsiveness to Photoperiod. *Plant Cell*. 23(1):147–161.

Heisler, M.G., Ohno, C., Das, P., Sieber, P., Reddy, G.V., Long, J.A., & Meyerowitz, E.M. 2005. Patterns of Auxin Transport and Gene Expression during Primordium Development Revealed by Live Imaging of the Arabidopsis Inflorescence Meristem. *Curr. Biol.* 15(21):1899–1911.

Hellens, R.P., Moreau, C., Lin-Wang, K., Schwinn, K.E., Thomson, S.J., Fiers, M.W.E.J., Frew, T.J., Murray, S.R., Hofer, J.M.I., Jacobs, J.M.E., Davies, K.M., Allan, A.C., Bendahmane, A., Coyne, C.J., Timmerman-Vaughan, G.M., & Ellis, T.H.N. 2010. Identification of Mendel's White Flower Character. *PLoS ONE*. 5(10):e13230.

Hennig, L. & Derkacheva, M. 2009. Diversity of Polycomb group complexes in plants: same rules, different players? *Trends Genet.* 25(9):414–423.

Hibara, K., Isono, M., Mimura, M., Sentoku, N., Kojima, M., Sakakibara, H., Kitomi, Y., Yoshikawa, T., Itoh, J., & Nagato, Y. 2016. Jasmonate regulates juvenile-to-adult phase transition in rice. *Development*. 143(18):3407–3416.

Hoch, H.C., Pratt, C., & Marx, G.A. 1980. Subepidermal Air Spaces: Basis for the Phenotypic Expression of the Argenteum Mutant of Pisum. *Am. J. Bot.* 67(6):905.

How Kit, A., Boureau, L., Stammitti-Bert, L., Rolin, D., Teyssier, E., & Gallusci, P. 2010. Functional analysis of SlEZ1 a tomato Enhancer of zeste (E(z)) gene demonstrates a role in flower development. *Plant Mol. Biol.* 74(3):201–213.

Huang, Y., Liu, C., Shen, W.-H., & Ruan, Y. 2011. Phylogenetic analysis and classification of the Brassica rapa SET-domain protein family. *BMC Plant Biol.* 11(1):1.

Hugueney, P., Bouvier, F., Badillo, A., d'Harlingue, A., Kuntz, M., & Camara, B. 1995. Identification of a plastid protein involved in vesicle fusion and/or membrane protein translocation. *Proc. Natl. Acad. Sci.* 92(12):5630–5634.

Huijser, P., Klein, J., Lönnig, W.E., Meijer, H., Saedler, H., & Sommer, H. 1992. Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene squamosa in Antirrhinum majus. *EMBO J.* 11(4):1239.

Huijser, P. & Schmid, M. 2011. The control of developmental phase transitions in plants. *Development*. 138(19):4117–4129.

Hunter, C., Sun, H., & Poethig, R.S. 2003. The Arabidopsis Heterochronic Gene ZIPPY Is an ARGONAUTE Family Member. *Curr. Biol.* 13(19):1734–1739.

Hunter, C.A. 2003. PAUSED Encodes the Arabidopsis Exportin-t Ortholog. *Plant Physiol.* 132(4):2135–2143.

Hutvagner, G. & Simard, M.J. 2008. Argonaute proteins: key players in RNA silencing. *Nat. Rev. Mol. Cell Biol.* 9(1):22–32.

Hyun, Y., Richter, R., Vincent, C., Martinez-Gallegos, R., Porri, A., & Coupland, G. 2016. Multi-layered Regulation of SPL15 and Cooperation with SOC1 Integrate Endogenous Flowering Pathways at the Arabidopsis Shoot Meristem. *Dev. Cell*. 37(3):254–266.

Irish, V.F. & Sussex, I.M. 1992. A fate map of the Arabidopsis embryonic shoot apical meristem. *Development*. 115(3):745–753.

Ivashuta, S., Imai, R., Uchiyama, K., Gau, M., & Shimamoto, Y. 2002. Changes in chloroplast FtsH-like gene during cold acclimation in alfalfa (*Medicago sativa*). *J. Plant Physiol.* 159:85–90.

Jagadeeswaran, G., Zheng, Y., Li, Y.-F., Shukla, L.I., Matts, J., Hoyt, P., Macmil, S.L., Wiley, G.B., Roe, B.A., Zhang, W., & Sunkar, R. 2009. Cloning and characterization of small RNAs from Medicago truncatula reveals four novel legume-specific microRNA families. *New Phytol.* 184(1):85–98.

James, S.A. & Bell, D.T. 2000. Influence of light availability on leaf structure and growth of two Eucalyptus globulus ssp. globulus provenances. *Tree Physiol.* 20(15):1007–1018.

James, S.A. & Bell, D.T. 2001. Leaf morphological and anatomical characteristics of heteroblastic Eucalyptus globulus ssp. globulus (Myrtaceae). *Aust. J. Bot.* 49(2):259–269.

Janska, H., Piechota, J., & Kwasniak, M. 2010. ATP-dependent proteases in biogenesis and maintenance of plant mitochondria. *Biochim. Biophys. Acta BBA - Bioenerg.* 1797(6–7):1071–1075.

Jiang, D., Wang, Y., Wang, Y., & He, Y. 2008. Repression of FLOWERING LOCUS C and FLOWERING LOCUS T by the Arabidopsis Polycomb Repressive Complex 2 Components. *PLoS ONE*. 3(10):e3404.

Jiao, Y., Wang, Y., Xue, D., Wang, J., Yan, M., Liu, G., Dong, G., Zeng, D., Lu, Z., Zhu, X., Qian, Q., & Li, J. 2010. Regulation of OsSPL14 by OsmiR156 defines ideal plant architecture in rice. *Nat. Genet.* 42(6):541–544.

Jofuku, K.D., Den Boer, B.G., Van Montagu, M., & Okamuro, J.K. 1994. Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. *Plant Cell*. 6(9):1211–1225.

Jordan, G.J., Potts, B.M., & Wiltshire, R.J. 1999. Strong, independent, quantitative genetic control of the timing of vegetative phase change and first flowering in Eucalyptus globulus ssp. globulus (Tasmanian Blue Gum). *Heredity*. 83(2):179–187.

Jung, J., Lee, S., Yun, J., Lee, M., & Park, C. 2014. The miR172 target TOE3 represses AGAMOUS expression during *Arabidopsis* floral patterning. *Plant Sci.* 215:29–38.

Jung, J.H., Ju, Y., Seo, P.J., Lee, J.H., & Park, C.H. 2012. The SOC1-SPL module integrates photoperiod and gibberellic acid signals to control flowering time in Arabidopsis: SOC1-SPL module in flowering promotion. *Plant J.* 69(4):577–588.

Jung, J.H., Seo, Y.H., Seo, P.J., Reyes, J.L., Yun, J., Chua, N.H., & Park, C.M. 2007. The GIGANTEA-Regulated MicroRNA172 Mediates Photoperiodic Flowering Independent of CONSTANS in Arabidopsis. *Plant Cell*. 19(9):2736–2748.

Kaló, P., Seres, A., Taylor, S.A., Jakab, J., Kevei, Z., Kereszt, A., Endre, G., Ellis, T.H.N., & Kiss, G.B. 2004. Comparative mapping between Medicago sativa and Pisum sativum. *Mol. Genet. Genomics*. 272(3):235–246.

Kankel, M.W., Ramsey, D.E., Stokes, T.L., Flowers, S.K., Haag, J.R., Jeddeloh, J.A., Riddle, N.C., Verbsky, M.L., & Richards, E.J. 2003. Arabidopsis MET1 cytosine methyltransferase mutants. *Genetics*. 163(3):1109–1122.

Kaplan, D. 1980. Heteroblastic leaf development in Acacia. Morphological and morphogenetic implications. *Cellule*. 73:137–203.

Karata, K., Inagawa, T., Wilkinson, A.J., Tatsuta, T., & Ogura, T. 1999. Dissecting the role of a conserved motif (the second region of homology) in the AAA family of ATPases Site-directed mutagenesis of the ATP-dependent protease FtsH. *J. Biol. Chem.* 274(37):26225–26232.

Katz, A., Oliva, M., Mosquna, A., Hakim, O., & Ohad, N. 2004. FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. *Plant J.* 37(5):707–719.

Kaur, S., Pembleton, L.W., Cogan, N.O., Savin, K.W., Leonforte, T., Paull, J., Materne, M., & Forster, J.W. 2012. Transcriptome sequencing of field pea and faba bean for discovery and validation of SSR genetic markers. *BMC Genomics*. 13(1):104.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*. 28(12):1647–1649.

Kim, J.J., Lee, J.H., Kim, W., Jung, H.S., Huijser, P., & Ahn, J.H. 2012. The microRNA156-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE3 Module Regulates Ambient Temperature-Responsive Flowering via FLOWERING LOCUS T in Arabidopsis. *PLANT Physiol.* 159(1):461–478.

Kim, W., Kim, H.E., Jun, A.R., Jung, M.G., Jin, S., Lee, J.H., & Ahn, J.H. 2016. Structural determinants of miR156a precursor processing in temperature-responsive flowering in Arabidopsis. *J. Exp. Bot.* 67(15):4659–4670.

Kim, Y.J., Zheng, B., Yu, Y., Won, S.Y., Mo, B., & Chen, X. 2011. The role of Mediator in small and long noncoding RNA production in Arabidopsis thaliana. *EMBO J.* 30(5):814–822.

Klein, J., Saedler, H., & Huijser, P. 1996. A new family of DNA binding proteins includes putative transcriptional regulators of theAntirrhinum majus floral meristem identity geneSQUAMOSA. *Mol. Gen. Genet. MGG*. 250(1):7–16.

Knight, T.A. 1795. Observations on the Grafting of Trees. In a Letter from Thomas Andrew Knight, Esq. to Sir Joseph Banks, Bart. P.R.S. *Philos. Transcations R. Sociey Lond.* 85:290–295.

Koenig, D., Bayer, E., Kang, J., Kuhlemeier, C., & Sinha, N. 2009. Auxin patterns Solanum lycopersicum leaf morphogenesis. *Development*. 136(17):2997–3006.

Köhler, C. & Grossniklaus, U. 2002. Epigenetic inheritance of expression states in plant development: the role of Polycomb group proteins. *Curr. Opin. Cell Biol.* 14(6):773–779.

Köhler, C. & Villar, C.B.R. 2008. Programming of gene expression by Polycomb group proteins. *Trends Cell Biol.* 18(5):236–243.

Kolodziejczak, M., Kolaczkowska, A., Szczesny, B., Urantowka, A., Knorpp, C., Kieleczawa, J., & Janska, H. 2002. A Higher Plant Mitochondrial Homologue of the Yeast m-AAA Protease: MOLECULAR CLONING, LOCALIZATION, AND PUTATIVE FUNCTION. *J. Biol. Chem.* 277(46):43792–43798.

Koressaar, T. & Remm, M. 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics*. 23(10):1289–1291.

Kozomara, A. & Griffiths-Jones, S. 2011. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* 39(suppl 1):D152–D157.

Kozomara, A. & Griffiths-Jones, S. 2014. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* 42(D1):D68–D73.

Kropat, J., Tottey, S., Birkenbihl, R.P., Depege, N., Huijser, P., & Merchant, S. 2005. A regulator of nutritional copper signaling in Chlamydomonas is an SBP domain protein that recognizes the GTAC core of copper response element. *Proc. Natl. Acad. Sci.* 102(51):18730–18735.

Krusell, L., Sato, N., Fukuhara, I., Koch, B.E.V., Grossmann, C., Okamoto, S., Oka-Kira, E., Otsubo, Y., Aubert, G., Nakagawa, T., Sato, S., Tabata, S., Duc, G., Parniske, M., Wang, T.L., Kawaguchi, M., &

Stougaard, J. 2011. The Clavata2 genes of pea and Lotus japonicus affect autoregulation of nodulation: Legume development and symbiosis. *Plant J.* 65(6):861–871.

Kujala, V. 1953. Felderbse bei welcher die ganze Blattspreite in Ranken umgewandelt ist. *Arch. Soc. Zool. Bot. Fenn.* 8:44–45.

Kurihara, Y. & Watanabe, Y. 2004. Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. *Proc. Natl. Acad. Sci.* 101(34):12753–12758.

Lafos, M., Kroll, P., Hohenstatt, M.L., Thorpe, F.L., Clarenz, O., & Schubert, D. 2011. Dynamic Regulation of H3K27 Trimethylation during Arabidopsis Differentiation. *PLoS Genet.* 7(4):e1002040.

Lamesch, P., Berardini, T.Z., Li, D., Swarbreck, D., Wilks, C., Sasidharan, R., Muller, R., Dreher, K., Alexander, D.L., Garcia-Hernandez, M., Karthikeyan, A.S., Lee, C.H., Nelson, W.D., Ploetz, L., Singh, S., Wensel, A., & Huala, E. 2012. The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. *Nucleic Acids Res.* 40(D1):D1202–D1210.

Lännenpää, M., Jänönen, I., Hölttä-Vuori, M., Gardemeister, M., Porali, I., & Sopanen, T. 2004. A new SBP-box gene BpSPL1 in silver birch (Betula pendula). *Physiol. Plant.* 120(3):491–500.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., & Higgins, D.G. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics*. 23(21):2947–2948.

Laubinger, S., Sachsenberg, T., Zeller, G., Busch, W., Lohmann, J.U., Rätsch, G., & Weigel, D. 2008. Dual roles of the nuclear cap-binding complex and SERRATE in pre-mRNA splicing and microRNA processing in Arabidopsis thaliana. *Proc. Natl. Acad. Sci.* 105(25):8795–8800.

Laucou, V., Haurogne, K., Ellis, N., & Rameau, C. 1998. Genetic mapping in pea. 1. RAPD-based genetic linkage map of Pisum sativum. *Theor. Appl. Genet.* 97(5):905–915.

Lauter, N., Kampani, A., Carlson, S., Goebel, M., & Moose, S.P. 2005. microRNA172 down-regulates glossy15 to promote vegetative phase change in maize. *Proc. Natl. Acad. Sci.* 102(26):9412–9417.

Lee, H., Yoo, S.J., Lee, J.H., Kim, W., Yoo, S.K., Fitzgerald, H., Carrington, J.C., & Ahn, J.H. 2010. Genetic framework for flowering-time regulation by ambient temperature-responsive miRNAs in Arabidopsis. *Nucleic Acids Res.* 38(9):3081–3093.

Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H., & Kim, V.N. 2004. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* 23(20):4051–4060.

Lee, Y.S., Lee, D.Y., Cho, L.H., & An, G. 2014. Rice miR172 induces flowering by suppressing OsIDS1 and SNB, two AP2 genes that negatively regulate expression of Ehd1 and florigens. *Rice*. 7(1):1–13.

Leonhard, K., Guiard, B., Pellecchia, G., Tzagoloff, A., Neupert, W., & Langer, T. 2000. Membrane protein degradation by AAA proteases in mitochondria: extraction of substrates from either membrane surface. *Mol. Cell*. 5(4):629–638.

Lester, D.R., Ross, J.J., Davies, P.J., & Reid, J.B. 1997. Mendel's stem length gene (Le) encodes a gibberellin 3 beta-hydroxylase. *Plant Cell*. 9(8):1435–1443.

Li, J. & Chen, X. 2003. PAUSED, a Putative Exportin-t, Acts Pleiotropically in Arabidopsis Development But Is Dispensable for Viability. *Plant Physiolo*. 132(4):1913–1924.

Libby, W.J. & Hood, J.V. 1976. Juvenility in hedged radiata pine. *Acta Hortic.* 56:91–98.

Lièvre, M., Granier, C., & Guédon, Y. 2016. Identifying developmental phases in the *Arabidopsis thaliana* rosette using integrative segmentation models. *New Phytol.* 210(4):1466–1478.

Liew, L.C., Hecht, V., Sussmilch, F.C., & Weller, J.L. 2014. The Pea Photoperiod Response Gene STERILE NODES Is an Ortholog of LUX ARRHYTHMO. *PLANT Physiol.* 165(2):648–657.

Lorenz, R., Bernhart, S.H., Zu Siederdissen, C.H., Tafer, H., Flamm, C., Stadler, P.F., & Hofacker, I.L. 2011. ViennaRNA Package 2.0. *Algorithms Mol. Biol.* 6(1):26.

Lu, C., Tej, S.S., Luo, S., Haudenschild, C.D., Meyers, B.C., & Green, P.J. 2005. Elucidation of the small RNA component of the transcriptome. *Science*. 309(5740):1567–1569.

Luo, M., Platten, D., Chaudhury, A., Peacock, W.J., & Dennis, E.S. 2009. Expression, Imprinting, and Evolution of Rice Homologs of the Polycomb Group Genes. *Mol. Plant*. 2(4):711–723.

Maher, C. 2006. Evolution of Arabidopsis microRNA families through duplication events. *Genome Res.* 16(4):510–519.

Manning, K., Tör, M., Poole, M., Hong, Y., Thompson, A.J., King, G.J., Giovannoni, J.J., & Seymour, G.B. 2006. A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nat. Genet.* 38(8):948–952.

Marco, A., MacPherson, J.I., Ronshaugen, M., & Griffiths-Jones, S. 2012. MicroRNAs from the same precursor have different targeting properties. *Silence*. 3(1):8.

Marcotrigiano, M. 1997. Chimeras and Variegation: Patterns of Deceit. *Hortic. Sci.* 32(5):773–784.

Martin, D.N., Proebsting, W.M., & Hedden, P. 1997. Mendel's dwarfing gene: cDNAs from the Le alleles and function of the expressed proteins. *Proc. Natl. Acad. Sci.* 94(16):8907–8911.

Marx, G.A. 1969. Linkage relations of *Af*. *Pisum Newsl.* 1:9–10.

Marx, G.A. 1986. Linkage of an aeromaculata mutant on chromosome 1. *Pisum Newsl*. 18:42–4.

Marx, G.A. & Hagedorn, D.J. 1962. Fasciation in pisum. *J. Hered.* 53(1):31–43.

Mathieu, J., Yant, L.J., Mürdter, F., Küttner, F., & Schmid, M. 2009. Repression of flowering by the miR172 target SMZ. *PLoS Biol*. 7(7):e1000148.

Matsoukas, I.G., Massiah, A.J., & Thomas, B. 2013. Starch metabolism and antiflorigenic signals modulate the juvenile-to-adult phase transition in Arabidopsis: Regulation of juvenility in Arabidopsis. *Plant Cell Environ.* 36(10):1802–1811.

May, P., Liao, W., Wu, Y., Shuai, B., Richard McCombie, W., Zhang, M.Q., & Liu, Q.A. 2013. The effects of carbon dioxide and temperature on microRNA expression in Arabidopsis development. *Nat. Commun.* 4:2145.

Medford, J.I., Behringer, F.J., Callos, J.D., & Feldmann, K.A. 1992. Normal and abnormal development in the Arabidopsis vegetative shoot apex. *Plant Cell*. 4(6):631–643.

Meier, I. & Brkljacic, J. 2009. The nuclear pore and plant development. *Curr. Opin. Plant Biol.* 12(1):87–95.

Meyers, B.C., Axtell, M.J., Bartel, B., Bartel, D.P., Baulcombe, D., Bowman, J.L., Cao, X., Carrington, J.C., Chen, X., & Green, P.J. 2008. Criteria for annotation of plant MicroRNAs. *Plant Cell Online*. 20(12):3186–3190.

Mica, E. 2006. Characterization of five microRNA families in maize. *J. Exp. Bot.* 57(11):2601–2612.

Mimura, M., Nagato, Y., & Itoh, J.-I. 2012. Rice PLASTOCHRON genes regulate leaf maturation downstream of the gibberellin signal transduction pathway. *Planta*. 235(5):1081–1089.

Moldavski, O., Levin-Kravets, O., Ziv, T., Adam, Z., & Prag, G. 2012. The Hetero-Hexameric Nature of a Chloroplast AAA+ FtsH Protease Contributes to Its Thermodynamic Stability. *PLoS ONE*. 7(4):e36008.

Moose, S.P. & Sisco, P.H. 1994. Glossy15 controls the epidermal juvenile-to-adult phase transition in maize. *Plant Cell Online*. 6(10):1343–1355.

Morea, E.G.O., Marques da Silva, E., Ferreira e Silva, G.F., Valente, G.T., Barrera Rojas, C.H., Vincentz, M., & Nogueira, F.T.S. 2016. Functional and evolutionary analyses of the miR156 and miR529 families in land plants. *BMC Plant Biol.* 16(1).

Mosquna, A., Katz, A., Decker, E.L., Rensing, S.A., Reski, R., & Ohad, N. 2009. Regulation of stem cell maintenance by the Polycomb protein FIE has been conserved during land plant evolution. *Development*. 136(14):2433–2444.

Müller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., & Simon, J.A. 2002. Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. *Cell*. 111(2):197–208.

Muller, J. & Verrijzer, P. 2009. Biochemical mechanisms of gene regulation by polycomb group protein complexes. *Curr. Opin. Genet. Dev.* 19(2):150–158.

Murfet, I.C. 1978. The flowering genes Lf, E, Sn and Hr in Pisum: their relationship with other genes, and their descriptions and type lines. *Pisum Newsl.* 10:48–52.

Murfet, I.C. 1990. Flowering genes in pea and their use in breeding. *Pisum Newsl.* 22:78–86.

Murfet, I.C. & Reid, J.B. 1993. Developmental mutants. , in: Peas - Genetics, Molecular Biology, and Biotechnology. CAB International: Wallingford, U.K. . CAB International: Wallingford, U.K., pp. 165–216.

Murfet, I.C. & Taylor, S.A. 2004. The *aero2 (aeromaculata2)* mutation in pea increases leaf flecking and complexity but, unlike aero1, does not promote flowering. *Pisum Genet.* 36.

Nair, S.K. & Tomar, S.M.S. 2001. Genetical and anatomical analyses of a leaf flecking mutant in Triticum aestivum L. *Euphytica*. 121(1):53–58.

Nair, S.K., Wang, N., Turuspekov, Y., Pourkheirandish, M., Sinsuwongwat, S., Chen, G., Sameri, M., Tagiri, A., Honda, I., Watanabe, Y., Kanamori, H., Wicker, T., Stein, N., Nagamura, Y., Matsumoto, T., & Komatsuda, T. 2010. Cleistogamous flowering in barley arises from the suppression of microRNA-guided HvAP2 mRNA cleavage. *Proc. Natl. Acad. Sci.* 107(1):490–495.

Ng, D., Wang, T., Chandrasekharan, M.B., Aramayo, R., Kertbundit, S., & Hall, T.C. 2007. Plant SET domain-containing proteins: Structure, function and regulation. *Biochim. Biophys. Acta BBA - Gene Struct. Expr.* 1769(5–6):316–329.

Njoku, E. 1956. The effect of defoliation on leaf shape in Ipomoea caerulea. *New Phytol.* 55(2):213–228.

Nozawa, M., Miura, S., & Nei, M. 2012. Origins and Evolution of MicroRNA Genes in Plant Species. *Genome Biol. Evol.* 4(3):230–239.

Ogura, T., Whiteheart, S.W., & Wilkinson, A.J. 2004. Conserved arginine residues implicated in ATP hydrolysis, nucleotide-sensing, and inter-subunit interactions in AAA and AAA+ ATPases. *J. Struct. Biol.* 146(1–2):106–112.

Ogura, T. & Wilkinson, A.J. 2001. AAA+ superfamily ATPases: common structure–diverse function. *Genes Cells*. 6(7):575–597.

Orkwiszewski, J.A. & Poethig, R.S. 2000. Phase identity of the maize leaf is determined after leaf initiation. *Proc. Natl. Acad. Sci.* 97(19):10631–10636.

O'Rourke, J.A., Bolon, Y.-T., Bucciarelli, B., & Vance, C.P. 2014. Legume genomics: understanding biology through DNA and RNA sequencing. *Ann. Bot.* 113(7):1107–1120.

Padmanabhan, M.S., Ma, S., Burch-Smith, T.M., Czymmek, K., Huijser, P., & Dinesh-Kumar, S.P. 2013. Novel Positive Regulatory Role for the SPL6 Transcription Factor in the N TIR-NB-LRR Receptor-Mediated Plant Innate Immunity. *PLoS Pathog.* 9(3):e1003235.

Parizotto, E.A. 2004. In vivo investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA. *Genes Dev.* 18(18):2237–2242.

Park, W., Li, J., Song, R., Messing, J., & Chen, X. 2002. CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. *Curr. Biol.* 12(17):1484–1495.

Pasternak, T., Potters, G., Caubergs, R., & Jansen, M.A.K. 2005. Complementary interactions between oxidative stress and auxins control plant growth responses at plant, organ, and cellular level. *J. Exp. Bot.* 56(418):1991–2001.

Peragine, A. 2004. SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis. *Genes Dev.* 18(19):2368–2379.

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29(9):e45–e45.

Pien, S. & Grossniklaus, U. 2007. *Polycomb* group and *trithorax* group proteins in *Arabidopsis*. *Biochim. Biophys. Acta BBA - Gene Struct. Expr.* 1769(5–6):375–382.

Poethig, R.S. 1988. Heterochronic Mutations Affecting Shoot Development in Maize. *Genetics*. 119(4):959–973.

Poethig, R.S. 1990. Phase change and the Regulation of Shoot Morphogenesis in Plants. *Science*. 250(4983):923–930.

Poethig, R.S. 2013. Vegetative Phase change and Shoot Maturation in Plants. *Curr. Top. Dev. Biol.* 105:125–152.

Preston, J.C. & Hileman, L.C. 2010. SQUAMOSA-PROMOTER BINDING PROTEIN 1 initiates flowering in Antirrhinum majus through the activation of meristem identity genes: Flowering time in snapdragon. *Plant J.* 62(4):704–712.

Preston, J.C. & Hileman, L.C. 2013. Functional Evolution in the Plant SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) Gene Family. *Front. Plant Sci.* 4(80):1–13.

Preston, J.C., Jorgensen, S.A., Orozco, R., & Hileman, L.C. 2016. Paralogous SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes differentially regulate leaf initiation and reproductive phase change in petunia. *Planta*. 243(2):429–440.

Proveniers, M. 2013. Sugars speed up the circle of life. *Elife*. 2:e00625.

Rameau, C., Bodelin, C., Cadier, D., Grandjean, O., Miard, F., & Murfet, I.C. 1997. New ramosus mutants at loci Rms1, Rms3 and Rms4 resulting from the mutation breeding program at Versailles. *Pisum Genet.* 29:7–12.

Refli. 2002. The control of vegetative phase change in *Pisum sativum L.* University of Tasmania: Hobart.

Reinhardt, D., Pesce, E.-R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., & Kuhlemeier, C. 2003. Regulation of phyllotaxis by polar auxin transport. *Nature*. 426(6964):255–260.

Reinhart, B.J. 2002. MicroRNAs in plants. *Genes Dev.* 16(13):1616–1626.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B., & Bartel, D.P. 2002. Prediction of plant microRNA targets. *Cell*. 110(4):513–520.

Ridge, S., Sussmilch, F.C., Hecht, V.F., Vander Schoor, J.K., Lee, R., Aubert, G., Burstin, J., Macknight, R.C., & Weller, J.L. 2016. Identification of LATE BLOOMER2 as a CYCLING DOF FACTOR Homolog Reveals Conserved and Divergent Features of the Flowering Response to Photoperiod in Pea. *Plant Cell*. 28(10):2545–2559.

Robbins, W.J. 1957. Physiological Aspects of Aging in Plants. *Am. J. Bot.* 44(3):289.

Rogers, K. & Chen, X. 2013. Biogenesis, Turnover, and Mode of Action of Plant MicroRNAs. *Plant Cell*. 25(7):2383–2399.

Rogler, C.E. & Hackett, W.P. 1975. Phase change in Hedera helix: induction of the mature to juvenile phase change by gibberellin A3. *Physiol. Plant.* 34(2):141–147.

Rozen, S. & Skaletsky, H. 1999. Primer3 on the WWW for general users and for biologist programmers. , in: Bioinformatics Methods and Protocols, Methods in Molecular Biology. Humana Press: Totowa, NJ. . Humana Press: Totowa, NJ, pp. 365–386.

Rubio-Somoza, I., Zhou, C.-M., Confraria, A., Martinho, C., von Born, P., Baena-Gonzalez, E., Wang, J.-W., & Weigel, D. 2014. Temporal Control of Leaf Complexity by miRNA-Regulated Licensing of Protein Complexes. *Curr. Biol.* 24(22):2714–2719.

Sachs, T. 1969. Regneration Experiments on the Determination of the Form of Leaves. *Isr. J. Bot.* 18:21–30.

Saito, K., Yoshikawa, M., Yano, K., Miwa, H., Uchida, H., Asamizu, E., Sato, S., Tabata, S., Imaizumi-Anraku, H., Umehara, Y., Kouchi, H., Murooka, Y., Szczyglowski, K., Downie, J.A., Parniske, M., Hayashi, M., & Kawaguchi, M. 2007. NUCLEOPORIN85 Is Required for Calcium Spiking, Fungal and Bacterial Symbioses, and Seed Production in Lotus japonicus. *Plant Cell*. 19(2):610–624.

Sakamoto, W. 2003. Leaf-variegated mutations and their responsible genes in *Arabidopsis thaliana*. *Genes Genet. Syst.* 78(1):1–9.

Sakamoto, W., Miura, E., Kaji, Y., Okuno, T., Nishizono, M., & Ogura, T. 2004. Allelic characterization of the leaf-variegated mutation var2 identifies the conserved amino acid residues of FtsH that are important for ATP hydrolysis and proteolysis. *Plant Mol. Biol.* 56(5):705–716.

Sakamoto, W., Tamura, T., Hanba-Tomita, Y., & Murata, M. 2002. The VAR1 locus of Arabidopsis encodes a chloroplastic FtsH and is responsible for leaf variegation in the mutant alleles. *Genes Cells*. 7(8):769–780.

Salinas, M., Xing, S., Höhmann, S., Berndtgen, R., & Huijser, P. 2012. Genomic organization, phylogenetic comparison and differential expression of the SBP-box family of transcription factors in tomato. *Planta*. 235(6):1171–1184.

Sato, Y., Morita, R., Nishimura, M., Yamaguchi, H., & Kusaba, M. 2007. Mendel's green cotyledon gene encodes a positive regulator of the chlorophyll-degrading pathway. *Proc. Natl. Acad. Sci.* 104(35):14169–14174.

Schaffalitzky de Muckadell, M. 1954. Juvenile stages in woody plants. *Physiol. Plant.* 7(4):782–796.

Schindelin, J., Rueden, C.T., Hiner, M.C., & Eliceiri, K.W. 2015. The ImageJ ecosystem: An open platform for biomedical image analysis: T HE I MAGEJ E COSYSTEM. *Mol. Reprod. Dev.* 82(7–8):518–529.

Schmittgen, T.D., Lee, E.J., Jiang, J., Sarkar, A., Yang, L., Elton, T.S., & Chen, C. 2008. Real-time PCR quantification of precursor and mature microRNA. *Methods*. 44(1):31–38.

Schneider, C.A., Rasband, W.S., & Eliceiri, K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods*. 9(7):671–675.

Schonrock, N. 2006. Polycomb-group proteins repressthe floral activator AGL19 in the FLC-independent vernalization pathway. *Genes Dev.* 20(12):1667–1678.

Schubert, D., Clarenz, O., & Goodrich, J. 2005. Epigenetic control of plant development by Polycomb-group proteins. *Curr. Opin. Plant Biol.* 8(5):553–561.

Schubert, D., Primavesi, L., Bishopp, A., Roberts, G., Doonan, J., Jenuwein, T., & Goodrich, J. 2006. Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. *EMBO J.* 25(19):4638–4649.

Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M., & Weigel, D. 2005. Specific Effects of MicroRNAs on the Plant Transcriptome. *Dev. Cell*. 8(4):517–527.

Schwarz, S., Grande, A.V., Bujdoso, N., Saedler, H., & Huijser, P. 2008. The microRNA regulated SBP-box genes SPL9 and SPL15 control shoot maturation in Arabidopsis. *Plant Mol. Biol.* 67(1–2):183–195.

Seo, S., Okamoto, M., Iwai, T., Iwano, M., Fukui, K., Isogai, A., Nakajima, N., & Ohashi, Y. 2000. Reduced levels of chloroplast FtsH protein in tobacco mosaic virus–infected tobacco leaves accelerate the hypersensitive reaction. *Plant Cell*. 12(6):917–932.

Shikata, M., Yamaguchi, H., Sasaki, K., & Ohtsubo, N. 2012. Overexpression of Arabidopsis miR157b induces bushy architecture and delayed phase transition in Torenia fournieri. *Planta*. 236(4):1027–1035.

Sidorova, K. & Uzhintseva, L. 1995. Analysis of induced supaeromaculata mutants in pea. *Russ. J. Genet.* 31:1008–1009.

Singer, S., Sollinger, J., Maki, S., Fishbach, J., Short, B., Reinke, C., Fick, J., Cox, L., McCall, A., & Mullen, H. 1999. Inflorescence architecture: A developmental genetics approach. *Bot. Rev.* 65(4):385–410.

Sinjushin, A.A. & Gostimskii, S.A. 2008. Genetic control of fasciation in pea (Pisum sativum L.). *Russ. J. Genet.* 44(6):702–708.

Smakowska, E., Blaszczyk, R.S., Czarna, M., Kolodziejczak, M., Owczarek, M.K., Parys, K., Funk, C., & Janska, H. 2016. Lack of FTSH4 protease affects protein carbonylation, mitochondrial morphology and phospholipid content in mitochondria of Arabidopsis: new insights into a complex interplay. *Plant Physiol.* 171(4):2516–2535.

Song, Q.-X., Liu, Y.-F., Hu, X.-Y., Zhang, W.-K., Ma, B., Chen, S.-Y., & Zhang, J.-S. 2011. Identification of miRNAs and their target genes in developing soybean seeds by deep sequencing. *BMC Plant Biol.* 11(1):5.

Song, S.-K. & Clark, S.E. 2005. POL and related phosphatases are dosage-sensitive regulators of meristem and organ development in Arabidopsis. *Dev. Biol.* 285(1):272–284.

Spillane, C., Schmid, K.J., Laoueill?-Duprat, S., Pien, S., Escobar-Restrepo, J.-M., Baroux, C., Gagliardini, V., Page, D.R., Wolfe, K.H., & Grossniklaus, U. 2007. Positive darwinian selection at the imprinted MEDEA locus in plants. *Nature*. 448(7151):349–352.

Springer, N.M., Danilevskaya, O.N., Hermon, P., Helentjaris, T.G., Phillips, R.L., Kaeppler, H.F., & Kaeppler, S.M. 2002. Sequence Relationships, Conserved Domains, and Expression Patterns for Maize Homologs of the Polycomb Group Genes E(z), esc, and E(Pc). *PLANT Physiol.* 128(4):1332–1345.

Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26(1–2):31–43.

Stief, A., Altmann, S., Hoffmann, K., Pant, B.D., Scheible, W.R., & Baurle, I. 2014. Arabidopsis miR156 Regulates Tolerance to Recurring Environmental Stress through SPL Transcription Factors. *Plant Cell*. 26(4):1792–1807.

Stone, J.M., Liang, X., Nekl, E.R., & Stiers, J.J. 2005. Arabidopsis AtSPL14, a plant-specific SBP-domain transcription factor, participates in plant development and sensitivity to fumonisin B1: AtSPL14 functions in plant development. *Plant J.* 41(5):744–754.

Sussex, I.M. & Clutter, M.E. 1960. A study of the effect of externally supplied sucrose on the morphology of excised fern leaves in vitro. *Phytomorphology*. 10(1):87–99.

Sussmilch, F.C., Berbel, A., Hecht, V., Vander Schoor, J.K., Ferrándiz, C., Madueño, F., & Weller, J.L. 2015. Pea *VEGETATIVE2* Is an *FD* Homolog That Is Essential for Flowering and Compound Inflorescence Development. *Plant Cell*. 27(4):1046–1060.

Szittya, G., Moxon, S., Santos, D.M., Jing, R., Fevereiro, M.P., Moulton, V., & Dalmay, T. 2008. High-throughput sequencing of Medicago truncatula short RNAs identifies eight new miRNA families. *BMC Genomics*. 9(1):593.

Tadege, M., Wen, J., He, J., Tu, H., Kwak, Y., Eschstruth, A., Cayrel, A., Endre, G., Zhao, P.X., Chabaud, M., Ratet, P., & Mysore, K.S. 2008. Large-scale insertional mutagenesis using the Tnt1 retrotransposon in the model legume Medicago truncatula. *Plant J.* 54(2):335–347.

Tayeh, N., Aluome, C., Falque, M., Jacquin, F., Klein, A., Chauveau, A., Bérard, A., Houtin, H., Rond, C., Kreplak, J., Boucherot, K., Martin, C., Baranger, A., Pilet-Nayel, M.L., Warkentin, T.D., Brunel, D., Marget, P., Le Paslier, M.C., Aubert, G., & Burstin, J. 2015. Development of two major resources for pea genomics: the GenoPea 13.2K SNP Array and a high-density, high-resolution consensus genetic map. *Plant J.* 84(6):1257–1273.

Taylor, S.A. & Murfet, I.C. 2003. A supaeromaculata mutation affects heterochrony in pea. *Physiol. Plant.* 117(1):100–107.

Telfer, A., Bollman, K.M., & Poethig, R.S. 1997. Phase change and the regulation of trichome distribution in Arabidopsis thaliana. *Development*. 124:645–654.

Telfer, A. & Poethig, R.S. 1994. Leaf Development in *Arabidopsis*. , in: *Arabidopsis*. Cold Spring Harbor Press: Cold Spring Harbor, NY. . Cold Spring Harbor Press: Cold Spring Harbor, NY, pp. 379–401.

Telfer, A. & Poethig, R.S. 1998. HASTY: a gene that regulates the timing of shoot maturation in Arabidopsis thaliana. *Development*. 125(10):1889–1898.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., & Higgins, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25(24):4876–4882.

Trievel, R.C., Beach, B.M., Dirk, L., Houtz, R.L., & Hurley, J.H. 2002. Structure and catalytic mechanism of a SET domain protein methyltransferase. *Cell*. 111(1):91–103.

Tripathi, R.K., Goel, R., Kumari, S., & Dahuja, A. 2017. Genomic organization, phylogenetic comparison, and expression profiles of the SPL family genes and their regulation in soybean. *Dev. Genes Evol.*

Troll, W. 1939. Vergleichende Morphologie der hoeheren Pflanzen. , in: 1: Vegetationsorgane. 2. Teil. Berlin, Borntraeger. . Berlin, Borntraeger.

Truskina, J. & Vernoux, T. 2018. The growth of a stable stationary structure: coordinating cell behavior and patterning at the shoot apical meristem. *Curr. Opin. Plant Biol.* 41:83–88.

Tsai, C.-H., Miller, A., Spalding, M., & Rodermel, S. 1997. Source strength regulates an early phase transition of tobacco shoot morphogenesis. *Plant Physiol.* 115(3):907–914.

Untergasser, A., Cutcutache, I., Koressaar, T., Faircloth, B.C., Remm, M., & Rozen, S. 2012. Primer3 - new capabilities and interfaces. *Nucleic Acids Res.* 40(15):e115–e115.

Urantowka, A., Knorpp, C., Olczak, T., Kolodziejczak, M., & Janska, H. 2005. Plant Mitochondria Contain at Least Two i-AAA-like Complexes. *Plant Mol. Biol.* 59(2):239–252.

Usami, T., Horiguchi, G., Yano, S., & Tsukaya, H. 2009. The more and smaller cells mutants of Arabidopsis thaliana identify novel roles for SQUAMOSA PROMOTER BINDING PROTEIN-LIKE genes in the control of heteroblasty. *Development*. 136(6):955–964.

Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E.F., & Hellens, R.P. 2007. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant Methods*. 3(1):12.

Varshney, R.K., Song, C., Saxena, R.K., Azam, S., Yu, S., Sharpe, A.G., Cannon, S., Baek, J., Rosen, B.D., Tar'an, B., Millan, T., Zhang, X., Ramsay, L.D., Iwata, A., Wang, Y., Nelson, W., Farmer, A.D., Gaur, P.M., Soderlund, C., Penmetsa, R.V., Xu, C., Bharti, A.K., He, W., Winter, P., Zhao, S., Hane, J.K., Carrasquilla-Garcia, N., Condie, J.A., Upadhyaya, H.D., Luo, M.C., Thudi, M., Gowda, C.L.L., Singh, N.P., Lichtenzveig, J., Gali, K.K., Rubio, J., Nadarajan, N., Dolezel, J., Bansal, K.C., Xu, X., Edwards, D., Zhang, G., Kahl, G., Gil, J., Singh, K.B., Datta, S.K., Jackson, S.A., Wang, J., & Cook, D.R. 2013. Draft genome sequence of chickpea (Cicer arietinum) provides a resource for trait improvement. *Nat. Biotechnol.* 31(3):240–246.

Vaucheret, H. 2004. The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev.* 18(10):1187–1197.

Vaughn, K.C. & Wilson, K.G. 1981. Development of Air Blisters in Pilea cadierei Gagnep. and Guillaumin. *Ann. Bot.* 48(4):467–472.

Vendemiatti, E., Zsogon, A., Felipe, G., & Silva, F. 2017. Loss of type-IV glandular trichomes is a heterochronic trait in tomato and can be reverted by promoting juvenility. *Plant Sci.* 259:35–47.

Voinnet, O. 2009. Origin, Biogenesis, and Activity of Plant MicroRNAs. *Cell*. 136(4):669–687.

Wagner, R., Aigner, H., & Funk, C. 2012. FtsH proteases located in the plant chloroplast. *Physiol. Plant.* 145(1):203–214.

Wagner, R., von Sydow, L., Aigner, H., Netotea, S., Brugière, S., Sjögren, L., Ferro, M., Clarke, A., & Funk, C. 2016. Deletion of FtsH11 protease has impact on chloroplast structure and function in *Arabidopsis thaliana* when grown under continuous light: Impact of FtsH11 in *Arabidopsis thaliana*. *Plant Cell Environ.* 39(11):2530–2544.

Wahl, V., Ponnu, J., Schlereth, A., Arrivault, S., Langenecker, T., Franke, A., Feil, R., Lunn, J.E., Stitt, M., & Schmid, M. 2013. Regulation of Flowering by Trehalose-6-Phosphate Signaling in Arabidopsis thaliana. *Science*. 339(6120):704–707.

Wang, H. & Wang, H. 2015. The miR156/SPL Module, a Regulatory Hub and Versatile Toolbox, Gears up Crops for Enhanced Agronomic Traits. *Mol. Plant*. 8(5):677–688.

Wang, J.W., Czech, B., & Weigel, D. 2009. miR156-Regulated SPL Transcription Factors Define an Endogenous Flowering Pathway in Arabidopsis thaliana. *Cell*. 138(4):738–749.

Wang, J.W., Park, M.Y., Wang, L.J., Koo, Y., Chen, X.Y., Weigel, D., & Poethig, R.S. 2011. MiRNA Control of Vegetative Phase Change in Trees. *PLoS Genet.* 7(2):e1002012.

Wang, J.W., Schwab, R., Czech, B., Mica, E., & Weigel, D. 2008. Dual Effects of miR156-Targeted SPL Genes and CYP78A5/KLUH on Plastochron Length and Organ Size in Arabidopsis thaliana. *Plant Cell*. 20(5):1231–1243.

Wang, S., Wu, K., Yuan, Q., Liu, X., Liu, Z., Lin, X., Zeng, R., Zhu, H., Dong, G., Qian, Q., Zhang, G., & Fu, X. 2012. Control of grain size, shape and quality by OsSPL16 in rice. *Nat. Genet.* 44(8):950–954.

Wang, Y., Wang, Z., Amyot, L., Tian, L., Xu, Z., Gruber, M.Y., & Hannoufa, A. 2015. Ectopic expression of miR156 represses nodulation and causes morphological and developmental changes in Lotus japonicus. *Mol. Genet. Genomics*. 290(2):471–484.

Wang, Z., Gerstein, M., & Snyder, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10(1):57–63.

Wareing, P.F. 1959. Problems of juvenility and flowering in trees. *Bot. J. Linn. Soc.* 56(366):282–289.

Wei, S., Gruber, M.Y., Yu, B., Gao, M.J., Khachatourians, G.G., Hegedus, D.D., Parkin, I.A., & Hannoufa, A. 2012. Arabidopsis mutant sk156 reveals complex regulation of SPL15 in a miR156-controlled gene network. *BMC Plant Biol.* 12(1):169.

Weller, J.L. 1997. Update on the genetics of flowering. *Pisum Genet.* 39:1–8.

Weller, J.L., Foucher, F., Hecht, V., & Rameau, C. 2003. New directions for the genetics of flowering in pea. *Flower. Newsl.* 36:14–24.

Weller, J.L., Liew, L.C., Hecht, V.F.G., Rajandran, V., Laurie, R.E., Ridge, S., Wenden, B., Schoor, J.K.V., Jaminon, O., Blassiau, C., Dalmais, M., Rameau, C., Bendahmane, A., Macknight, R.C., & Lejeune-

Hénaut, I. 2012. A conserved molecular basis for photoperiod adaptation in two temperate legumes. *Proc. Natl. Acad. Sci.* 109(51):21158–21163.

Weller, J.L., Reid, J.B., Taylor, S.A., & Murfet, I.C. 1997. The genetic control of flowering in pea. *Trends Plant Sci.* 2(11):412–418.

Wiltshire, R.J.E., Murfet, I.C., & Reid, J.B. 1994. The genetic control of heterochrony: Evidence from developmental mutants of Pisum sativum L. *J. Evol. Biol.* 7(4):447–465.

Wiltshire, R.J.E., Potts, B.M., & Reid, J.B. 1991. A paedomorphocline in Eucalyptus: Natural variation in the E. risdonii/E. tenuiramis complex. *Aust. J. Bot.* 39(6):545–566.

Wiltshire, R.J.E. & Reid, J.B. 1992. The pattern of juvenility within Eucalyptus tenuiramis Miq. saplings. , in: Mass Production Technology for Genetically Improved Fast Growing Forest Tree Species. Nangis-Paris: Association Forest Cellulose: AFOCEL-IUFRO Symposium, Bordeaux. . Nangis-Paris: Association Forest Cellulose: AFOCEL-IUFRO Symposium, Bordeaux, pp. 37–49.

Wiltshire, R.J.E., Reid, J.B., & Potts, B.M. 1998. Genetic Control of Reproductive and Vegetative Phase Change in the Eucalyptus risdonii–E. tenuiramis Complex. *Aust. J. Bot.* 46(1):45.

Wollmann, H. & Berger, F. 2015. Epigenetic Modifications at Developmental Transitions in Arabidopsis. , in: Pontes, O., Jin, H. (Eds.), Nuclear Functions in Plant Transcription, Signaling and Development. Springer New York: New York, NY. . Springer New York: New York, NY, pp. 119–131.

Wu, G., Park, M.Y., Conway, S.R., Wang, J.-W., Weigel, D., & Poethig, R.S. 2009. The Sequential Action of miR156 and miR172 Regulates Developmental Timing in Arabidopsis. *Cell*. 138(4):750–759.

Wu, G. & Poethig, R.S. 2006. Temporal regulation of shoot development in Arabidopsis thaliana by miR156 and its target SPL3. *Development*. 133(18):3539–3547.

Xie, K. 2006. Genomic Organization, Differential Expression, and Interaction of SQUAMOSA Promoter-Binding-Like Transcription Factors and microRNA156 in Rice. *Plant Physiol.* 142(1):280–293.

Xie, Z. 2005. Expression of Arabidopsis MIRNA Genes. *PLANT Physiol.* 138(4):2145–2154.

Xie, Z., Allen, E., Wilken, A., & Carrington, J.C. 2005. DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in Arabidopsis thaliana. *Proc. Natl. Acad. Sci. U. S. A.* 102(36):12984–12989.

Xu, M., Hu, T., Smith, M.R., & Poethig, R.S. 2015. Epigenetic Regulation of Vegetative Phase Change in Arabidopsis. *Plant Cell*. 28(1):28–41.

Xu, M., Hu, T., Zhao, J., Park, M.Y., Earley, K.W., Wu, G., Yang, L., & Poethig, R.S. 2016. Developmental Functions of miR156-Regulated SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) Genes in Arabidopsis thaliana. *PLOS Genet.* 12(8):e1006263.

Xu, Y., Guo, C., Zhou, B., Li, C., Wang, H., Zheng, B., Ding, H., Zhu, Z., Peragine, A., Cui, Y., Poethig, R.S., & Wu, G. 2016. Regulation of vegetative phase change by SWI2/SNF2 chromatin remodeling ATPase BRAHMA. *Plant Physiol.* 172(4):2416–2428.

Xue, X.Y., Zhao, B., Chao, L.U., Chen, D.Y., Cui, W.R., Mao, Y.B., Wang, L.J., & Chen, X.Y. 2014. Interaction between Two Timing MicroRNAs Controls Trichome Distribution in Arabidopsis. *PLoS Genet.* 10(4):e1004266.

Yamada-Inagawa, T., Okuno, T., Karata, K., Yamanaka, K., & Ogura, T. 2003. Conserved Pore Residues in the AAA Protease FtsH Are Important for Proteolysis and Its Coupling to ATP Hydrolysis. *J. Biol. Chem.* 278(50):50182–50187.

Yamaguchi, A., Wu, M.-F., Yang, L., Wu, G., Poethig, R.S., & Wagner, D. 2009. The MicroRNA-Regulated SBP-Box Transcription Factor SPL3 Is a Direct Upstream Activator of LEAFY, FRUITFULL, and APETALA1. *Dev. Cell*. 17(2):268–278.

Yamasaki, K., Kigawa, T., Inoue, M., Tateno, M., Yamasaki, T., Yabuki, T., Aoki, M., Seki, E., Matsuda, T., Nunokawa, E., Ishizuka, Y., Terada, T., Shirouzu, M., Osanai, T., Tanaka, A., Seki, M., Shinozaki, K., & Yokoyama, S. 2004. A Novel Zinc-binding Motif Revealed by Solution Structures of DNA-binding Domains of Arabidopsis SBP-family Transcription Factors. *J. Mol. Biol.* 337(1):49–63.

Yamashino, T., Yamawaki, S., Hagui, E., Ishida, K., Ueoka-Nakanishi, H., Nakamichi, N., & Mizuno, T. 2013. Clock-Controlled and FLOWERING LOCUS T (FT)-Dependent Photoperiodic Pathway in *Lotus japonicus* II: Characterization of a MicroRNA Implicated in the Control of Flowering Time. *Biosci. Biotechnol. Biochem.* 77(6):1179–1185.

Yang, L., Conway, S.R., & Poethig, R.S. 2010. Vegetative phase change is mediated by a leaf-derived signal that represses the transcription of miR156. *Development*. 138(2):245–249.

Yang, L., Conway, S.R., & Poethig, R.S. 2011. Vegetative phase change is mediated by a leaf-derived signal that represses the transcription of miR156. *Development*. 138(2):245–249.

Yang, L., Liu, Z., Lu, F., Dong, A., & Huang, H. 2006. SERRATE is a novel nuclear regulator in primary microRNA processing in Arabidopsis. *Plant J.* 47(6):841–850.

Yang, L., Xu, M., Koo, Y., He, J., & Poethig, R.S. 2013. Sugar promotes vegetative phase change in Arabidopsis thaliana by repressing the expression of MIR156A and MIR156C. *Elife*. 2:e00260.

Yang, Z., Wang, X., Gu, S., Hu, Z., Xu, H., & Xu, C. 2008. Comparative study of SBP-box gene family in Arabidopsis and rice. *Gene*. 407(1–2):1–11.

Yin, J. -w. & Wang, G. 2014. The Mediator complex: a master coordinator of transcription and cell lineage development. *Development*. 141(5):977–987.

Yin, Z., Li, C., Han, X., & Shen, F. 2008. Identification of conserved microRNAs and their target genes in tomato (Lycopersicon esculentum). *Gene*. 414(1):60–66.

Yoshikawa, M. 2005. A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. *Genes Dev.* 19(18):2164–2175.

Yoshikawa, T., Ozawa, S., Sentoku, N., Itoh, J.I., Nagato, Y., & Yokoi, S. 2013. Change of shoot architecture during juvenile-to-adult phase transition in soybean. *Planta*. 238(1):229–237.

Yu, F., Park, S., & Rodermel, S.R. 2004. The *Arabidopsis* FtsH metalloprotease gene family: interchangeability of subunits in chloroplast oligomeric complexes. *Plant J.* 37(6):864–876.

Yu, L.P., Miller, A.K., & Clark, S.E. 2003. POLTERGEIST encodes a protein phosphatase 2C that regulates CLAVATA pathways controlling stem cell identity at Arabidopsis shoot and flower meristems. *Curr. Biol.* 13(3):179–188.

Yu, N., Cai, W.J., Wang, S., Shan, C.M., Wang, L.J., & Chen, X.Y. 2010. Temporal Control of Trichome Distribution by MicroRNA156-Targeted SPL Genes in Arabidopsis thaliana. *Plant Cell*. 22(7):2322–2335.

Yu, S., Cao, L., Zhou, C.M., Zhang, T.Q., Lian, H., Sun, Y., Wu, J., Huang, J., Wang, G., & Wang, J.W. 2013. Sugar is an endogenous cue for juvenile-to-adult phase transition in plants. *eLife*. 2.

Yu, S., Galvao, V.C., Zhang, Y.-C., Horrer, D., Zhang, T.Q., Hao, Y.H., Feng, Y.Q., Wang, S., Schmid, M., & Wang, J.W. 2012. Gibberellin Regulates the Arabidopsis Floral Transition through miR156-Targeted SQUAMOSA PROMOTER BINDING-LIKE Transcription Factors. *Plant Cell*. 24(8):3320–3332.

Zhang, B., Pan, X., & Stellwag, E.J. 2008. Identification of soybean microRNAs and their targets. *Planta*. 229(1):161–182.

Zhang, B., Xu, W., Liu, X., Mao, X., Li, A., Wang, J., Chang, X., Zhang, X., & Jing, R. 2017. Functional conservation and divergence among homoeologs of TaSPL20 and TaSPL21, two SBP-box genes governing yield-related traits in hexaploid wheat. *Plant Physiol.* 174(2):1177–1191.

Zhang, S., Wu, J., Yuan, D., Zhang, D., Huang, Z., Xiao, L., & Yang, C. 2014. Perturbation of Auxin Homeostasis Caused by Mitochondrial FtSH4 Gene-Mediated Peroxidase Accumulation Regulates Arabidopsis Architecture. *Mol. Plant*. 7(5):856–873.

Zhang, W., Li, B., & Yu, B. 2016. Genome-wide identification, phylogeny and expression analysis of the SBP-box gene family in maize (Zea mays). *J. Integr. Agric.* 15(1):29–41.

Zhang, X., Zou, Z., Zhang, J., Zhang, Y., Han, Q., Hu, T., Xu, X., Liu, H., Li, H., & Ye, Z. 2011. Over-expression of sly-miR156a in tomato results in multiple vegetative and reproductive trait alterations and partial phenocopy of the *sft* mutant. *FEBS Lett.* 585(2):435–439.

Zhang, Y., Schwarz, S., Saedler, H., & Huijser, P. 2007. SPL8, a local regulator in a subset of gibberellin-mediated developmental processes in Arabidopsis. *Plant Mol. Biol.* 63(3):429–439.

Zhou, C., Zhang, TQ, Wang X, Yu S, Lian H, Tang H, Feng ZY, Zozomova-Lihova J, & Wang JW. 2013. Molecular Basis of Age-Dependent Vernalization in *Cardamine flexuosa*. *Science*. 340(6136):1097–1100.

Zhu, Q.H. & Helliwell, C.A. 2010. Regulation of flowering time and floral patterning by miR172. *J. Exp. Bot.* 62(2):487–495.

Zimmerman, R.H., Hackett, W.P., & Pharis, R.P. 1985. Hormonal Aspects of Phase Change and Precocious Flowering. , in: Hormonal Regulation of Development III. Springer: Berlin Heidelberg. . Springer: Berlin Heidelberg., pp. 79–115.